Polimorfismo en el color floral y especiación en *Lysimachia arvensis*

Tesis doctoral

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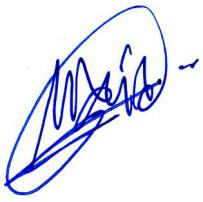

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A María José
A Paco
A Julio

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AGRADECIMIENTOS

Antonio Machado, allá por 1917 escribía en la sección “proverbios y cantares” de su mítica obra, Campos de castilla:

Caminante, son tus huellas
el camino y nada más;
Caminante, no hay camino,
se hace camino al andar.
Al andar se hace el camino,
y al volver la vista atrás
se ve la senda que nunca
se ha de volver a pisar.
Caminante no hay camino
sino estelas en la mar.

Este poema lleno de simbolismo ha sido una de las máximas que han guionizado mi vida desde hace años. Especialmente en el ámbito que ahora nos ocupa. Para mí, la tesis siempre ha sido un camino, un camino por el que discurrimos en busca de respuestas, y son, esas respuestas, las que van guiando ese camino. Por suerte para mí, durante ese caminar me he encontrado con numerosas personas que han interaccionado a mi discurrir. Su interacción ha oscilado desde una puntual, pero imprescindible, dosis de aliento hasta una guía experta, constante y perseverante, contribuyendo a la culminación de este complejo, pero gratificante, proyecto. Son tantos los apoyos recibidos, que probablemente no tenga espacio para ello, aunque intentaré no olvidar a nadie, si ocurriera, discúlpeme el no aludido.

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RESUMEN

Uno de los caracteres que promueven la selección divergente es el polimorfismo del color que se define como la presencia en una especie de dos o más morfos de color, genéticamente determinados e interfértiles en las mismas poblaciones. En plantas, el polimorfismo del color floral es un fenómeno ampliamente distribuido, pero poco frecuente, que se debe principalmente a la presencia de antocianinas. El polimorfismo del color ha sido definido como un "carácter mágico", es decir, un carácter codificado por genes que están sujetos a selección divergente y que afectan pleiotrópicamente al aislamiento reproductivo, por lo que puede desencadenar un proceso de especiación. Aunque para que el proceso de especiación se complete es necesario el desarrollo de un considerable número de barreras pre- y postcigóticas entre las especies incipientes. A este respecto, hay que señalar que el color floral puede no ser el carácter que desencadene un proceso de especiación, pero puede ser fundamental como mecanismo de "refuerzo" creando barreras de aislamiento entre esas especies. El objetivo principal de esta tesis doctoral es conocer si el polimorfismo de color floral puede ser el desencadenante de un proceso de especiación en *Lysimachia arvensis* (L.) U. Manns & Anderb, una especie anual con plantas con flores azules o rojas. Para llevar a cabo este objetivo hemos estudiado diversos aspectos ecológicos, reproductivos y moleculares de esta especie, como la segregación del color floral, el papel de los polinizadores como agentes de selección, la fenología de los morfos de color, su sistema de cruzamiento, la depresión por endogamia, la diversidad genética de las poblaciones y la filogenia del grupo de especies en las que se incluye *L. arvensis*. Hemos encontrado que tras el cruce entre morfos de color se origina una F1 homogénea con flores salmón y que aparece con muy poca frecuencia en las poblaciones naturales. Los espectros de reflectancia muestran que el morfo azul es el que contrasta más con el fondo verde de las hojas, y que los morfos salmón y rojo son poco diferenciables bajo el sistema visual de las abejas, sus principales polinizadores. Además, se ha observado la presencia de dos tipos consecutivos de hercogamia, lateral y vertical, un fenómeno muy raro en las angiospermas. Ambos tipos de hercogamia tienen un alto grado de heredabilidad, y difieren entre morfos. El morfo rojo presenta una marcada hercogamia lateral en el primer día de apertura floral y hercogamia reversa durante el segundo día, favoreciendo la autopolinización retardada. El azul mostró mucha más variabilidad con poblaciones más xenógamas con una marcada hercogamia lateral el primer día y hercogamia de aproximación después, mientras que otras mostraron poca hercogamia lateral y posteriormente de aproximación, lo que favorece la autogamia competitiva. Además hemos encontrado una disminución de la hercogamia en poblaciones simpátricas con

respecto a las alopátricas. Respecto a las barreras pre- y postcigóticas que pueden aparecer entre los dos morfos de color se han estudiado las ecogeográficas, fenológicas, dirigidas por los polinizadores, la precedencia polínica y las relacionadas con el cruce entre morfos (formación de frutos y semillas, germinación, viabilidad y supervivencia de las plántulas, producción de frutos de la F1 y F2). El aislamiento ecogeográfico, la precedencia del polen y el aislamiento por polinizadores fueron las barreras más importantes, contribuyendo a un aislamiento global entre morfos de un 0,7855.

Además, los morfos mostraron diferencias en la fenología de germinación y floración, siendo el morfo azul el que germinó primero y floreció más tempranamente. Los niveles de depresión por endogamia fueron altos para el morfo rojo (alrededor de 0,6) y bastante más bajos para el morfo azul (0,18-0,32), a pesar de lo cual la diversidad genética del morfo rojo fue muy baja en comparación con la del azul tanto en poblaciones Mediterráneas como no Mediterráneas. De hecho, ambos morfos mostraron una alta diferenciación genética entre ellos. Todos los resultados que hemos ido obteniendo sugieren una gran diferenciación entre los dos morfos de *L. arvensis* y una baja tasa de cruzamientos en poblaciones naturales. El estudio filogenético mostró un agrupamiento de los individuos de *L. arvensis* con independencia de su color floral cuando se utilizaron marcadores plástidiales. Sin embargo, los marcadores nucleares agruparon al morfo rojo con *L. monelli* y *L. foemina*, y en un clado diferente al morfo azul junto con *L. talaverae*. *L. arvensis* parece estar compuesto por dos linajes totalmente diferentes que pueden ser considerados bajo la categoría de especie. Proponemos el nombre de *L. arvensis* para el morfo rojo y *L. loefflingi*, nombre nuevo, para el morfo azul. El conjunto de resultados obtenidos en esta tesis doctoral indica que el color floral no ha sido el carácter que ha desencadenado el proceso de especiación en *L. arvensis*. Sin embargo, el hecho de que el color floral esté directamente implicado en importantes barreras precigóticas de aislamiento reproductivo entre estos dos taxones sugiere fuertemente que este rasgo podría estar funcionando como mecanismo de refuerzo, manteniendo ambas especies como entidades independientes.

ABSTRACT

Colour polymorphism is defined as the presence of two or more colour morphs, genetically determined and interfertile, within populations of a species. In plants, flower colour polymorphism is a widespread phenomenom but relatively infrequent and depends mainly of the presence of anthocyanins. Colour polymorphism has been defined as a “magic trait”, that is, a character encoded by genes that are subject to divergent selection that affects pleiotropically reproductive isolation, acting as a trigger for the

speciation process. An essential component of speciation is the evolution of reproductive isolation. In plants, reproductive isolation depends on the existence of a considerable number of pre- and post-zygotic barriers between the incipient species. However, sometime flower colour does not initiate the speciation process but it can strongly contribute to species separation by acting as a reinforcement mechanism. The main objective of this doctoral thesis is to know if the floral colour polymorphism can be unleashing of speciation process in *Lysimachia arvensis* (L.) U. Manns & Anderb., an annual forb with red or blue flowers. To do this,

we have carried out ecological, reproductive and molecular studies of *L. arvensis* such as floral colour segregation, plant phenology, breeding system and endogamy depression, genetic diversity of populations and the phylogeny of the *L. arvensis* group. We have found that inter-morph cross originates a homogeneous F1 with salmon-colour flowers, very infrequent in natural populations. The reflectance spectra of these morphs show that blue flowers markedly contrast with the background of the leaves. Bees, the main pollinators of this species, hardly distinguish between salmon and red flowers. In addition, flowers have two consecutive kinds of hercogamy, lateral and vertical, a very rare phenomenon in flowering plants. Both types of hercogamy have a high degree of heritability, and differ between morphs. The red morph showed a marked lateral hercogamy on the first anthesis day of the flowers and reverse hercogamy during the second day, favouring delayed selfing. The blue morph was much more variable, some populations had a marked lateral herkogamy and then, approach herkogamy while others had low lateral herkogamy and then, approach hercogamy, favouring competing selfing. Herkogamy traits decreased significantly when morphs occur in sympatry than in allopatry. In relation to the pre- and post-zygotic barriers between the two colour morphs, the ecogeographical, phenological, driven by pollinators, pollen precedence and those related to the between morph crossing (fruit- and seed-set, germination, viability and survival of progeny F1 and F2) were studied. The ecogeographical isolation, the pollen precedence and the isolation by pollinators were the most important barriers, contributing to a isolation of 0.7855.

In addition, the germination and flowering phenology differed between morphs occurring earlier in the blue morph. Inbreeding depression levels were high in the red morph (about 0.6) and much lower in he blue morph (0.18-0.32). Despite that, genetic diversity of the red morph was lower than that of the blue morph in both Mediterranean and non-Mediterranean populations. In fact, the blue and red morph showed a huge genetic differentiation among them. All the results obtained suggest a high differentiation and a low pollen flow between morphs. The phylogenetic study of *Lysimachia arvensis* clade grouped both colour morphs in the same clade when using plastid markers. However, the nuclear markers grouped the red morph together both *L. monelli* and *L. foemina*, and

the blue morph in a different clade with *L*. *talaverae*. These results strongly suggest that *L*. *arvensis* is composed by two different lineages that can be considered as different species. We propose the name of *L. arvensis* for the red morph and *L. loefflingi*, new name, for the blue morph. All results obtained in this doctoral thesis indicate that flower colour has not driven the speciation process in *L*. *arvensis*. However, the fact that flower colour is directly associated to important prezygotic barriers between both taxa strongly suggest its role as reinforcement mechanism, maintaining both lineages as independent entities.

1. Introducción.

Jiménez-López F.J.

INTRODUCCIÓN

Los patrones actuales de diversidad biológica resultan de las interacciones entre especiación, cambios en el rango de distribución y extinción. De estos procesos, la especiación es el único que genera nueva diversidad y consecuentemente la dinámica de los procesos de especiación constituye un tema central en biología evolutiva. Los procesos de especiación son particularmente frecuentes en las plantas con flores, siendo estas aventajadas únicamente por los insectos en cuanto a diversidad de especies; además, gran parte de la diversificación de dichas plantas ha ocurrido recientemente, generando ejemplos espectaculares de radiación adaptativa y de especiación en acción (Rieseberg & Willis 2007).

Un componente esencial de la especiación es la evolución del aislamiento reproductivo, esto es, la aparición de barreras reproductivas que impidan el flujo génico entre grupos de individuos (Dobzhansky 1937; Mayr 1942; Coyne & Orr 2004; Coyne 2007; Sobel & Chen 2014). Estas barreras deben de ser mantenidas en el tiempo para que los grupos afectados lleguen a ser entidades diferentes (Dobzhansky 1937; Mayr 1942; Coyne & Orr 2004), aunque se desconoce el grado de aislamiento reproductivo requerido (Widmer et al. 2009). En plantas, el aislamiento reproductivo depende de un considerable número de barreras pre- y postcigóticas y sus interacciones (Lowry et al. 2008; Widmer et al. 2009). Las barreras precigóticas incluyen diferencias geográficas y ecológicas, separación fenológica, diferencias en los polinizadores, apareamiento selectivo e interacciones entre el polen y el pistilo (Mayr 1963; Coyne & Orr 2004), mientras que las barreras postcigóticas pueden ser intrínsecas e incluir baja viabilidad híbrida o esterilidad (Dobzhansky 1937, Muller 1942), o extrínsecas como la inferioridad ecológica del híbrido y su bajo éxito reproductivo (Rundle et al. 2000; Schluter 2000). Aunque menos considerada, la autogamia puede ser una importante barrera precigótica que reduce el flujo génico entre grupos de individuos (Martin & Willis 2007; Levin 2010; Runquist & Moeller 2014). Los estudios más recientes concluyen que el aislamiento precigótico es mucho más importante que el postcigótico, por dos motivos: 1) porque, considerando cada barrera por separado, son más potentes que las postcigóticas, y 2) porque la contribución relativa de cada barrera es tanto mayor cuanto antes actúe en el ciclo de vida (Rieseberg & Willis 2007; Lowry et al. 2008). A pesar del gran interés que suscita la comprensión del aislamiento reproductivo, solo unos pocos estudios han profundizado en la contribución de las diferentes barreras al mismo. Sin embargo, para comprender el proceso de especiación es importante estudiar todas las barreras

reproductivas potenciales (Lowry et al. 2008), intentando explorarlas en distintos estadios a lo largo del proceso de divergencia que lleva a la especiación (Via 2009).

En los últimos años se han hecho importantes avances en la caracterización de los genes que contribuyen al aislamiento reproductivo entre poblaciones y que son vitales para desencadenar los procesos de especiación (Rieseberg & Willis 2007, Butlin & Ritchie 2001; Noor 2003; Rieseberg et al. 2004; Presgraves 2010). El conocimiento de los "genes de especiación" ofrece información vital para entender cómo se produce la divergencia (Orr et al. 2006). Hasta hace poco, el interés se centraba en los genes que contribuyen al aislamiento postcigótico, sin embargo, dada la importancia de las barreras precigóticas, actualmente los genes que contribuyen a crear este tipo de barreras se encuentran en el centro de atención de los biólogos evolutivos (Rieseberg & Blackman 2010).

La selección divergente es un importante mecanismo promotor de la especiación, tal como se ha probado en numerosos grupos de organismos (Funk et al. 2006; Nossil et al. 2009; Pfennig & Pfennig 2010) y puede originar diferenciación incluso con cierto nivel de flujo génico (Rosenblum 2005). En estos casos, la especiación se ve facilitada si los caracteres sujetos a selección divergente, u otros correlacionados genéticamente, afectan a la compatibilidad reproductiva o la probabilidad de apareamiento (Servedio et al. 2011). Los polinizadores son importantes agentes de selección que pueden promover la diversificación en plantas. Así, plantas polinizadas por polinizadores diferentes estarían aisladas reproductivamente y podrían estar sometidas a selección divergente; pero aún entre plantas que comparten polinizadores, diferencias en la frecuencia relativa de visitas de éstos y en su eficiencia como polinizadores pueden dar lugar a apareamientos no azarosos que disminuyen el flujo génico y contribuyen generar aislamiento reproductivo (Kay & Sargent 2009). Asimismo, la constancia floral (tendencia de los polinizadores a visitar secuencialmente flores de un determinado morfotipo; Waser 1986) también genera apareamientos no azarosos disminuyendo o incluso interrumpiendo el flujo génico entre morfotipos. En definitiva, los polinizadores como agentes de selección pueden fomentar la divergencia y como agentes de flujo génico pueden reducir o interrumpir el flujo génico (Waser 1986). En sentido opuesto, también pueden promover el flujo génico y la hibridación entre taxones previamente separados que entren en contacto (Kay & Sargent 2009).

El rol del polimorfismo de color en los procesos de especiación

Uno de los caracteres que promueven la selección divergente es el polimorfismo del color (Gray & McKinnon 2007; Forsman et al. 2008; McLean & Stuart-Fox 2014) que se define como la presencia en una especie de dos o más morfotipos de color,

genéticamente determinados e interfértiles. Para que el polimorfismo del color sea considerado estable, el morfotipo más raro debe estar en una frecuencia suficientemente alta como para que no sea resultado de una mutación recurrente (Huxley 1955; Levin & Brack 1995; Wu et al. 2013). Las variaciones de color se pueden encontrar entre individuos de una misma población o entre poblaciones (Wright 1978). Aunque el polimorfismo de color a menudo es considerado un carácter discreto, esta idea ha quedado cuestionada cuando se han realizado estudios exhaustivos de la variación fenotípica de este carácter, tanto en animales (Vercken & Clobert 2008) como en plantas (Whittall, et al. 2010; Carlson & Holsinger 2013; Narbona et al. 2014).

El polimorfismo del color, aunque poco frecuente, está representado en grupos taxonómicamente muy diversos (Hugall & Stuart-Fox 2012). En plantas, se ha documentado en al menos 21 familias y 32 géneros (Wheldale 1916; Rausher 2008; Narbona et al. 2018). El polimorfismo del color floral y su mantenimiento han suscitado gran interés en los últimos años (Subramanian & Rausher 2000; Irwin et al. 2003; Arista et al. 2013; Imbert et al. 2014; Ortiz et al. 2015). De hecho, el polimorfismo del color se considera un "carácter mágico", es decir, un carácter codificado por genes que están sujetos a selección divergente y que afectan pleiotrópicamente al aislamiento reproductivo (Servedio et al. 2011). En estos casos, la selección favorece a los diferentes morfotipos (posibles especies incipientes) que coexisten y evolucionan hacia diferentes óptimos, bien dentro de una población mixta o en diferentes poblaciones con algún flujo génico (Sobel et al. 2010). Solo existe un caso en el que un cambio en el color floral parece haber originado especiación (Bradshaw & Schemske 2003). El cambio de color de pétalos rosas de *Mimulus lewisii* (polinizado por abejas) al rojo de *M. cardinalis* (polinizado por colibríes) está controlado por un único *locus*. La manipulación de este *locus* en *M. lewisii* origina un cambio de color que provoca que las flores sean visitadas principalmente por colibríes, disminuyendo pleiotrópicamente las visitas de abejas, lo que conllevaría un aislamiento reproductivo por polinizadores. Este caso sirve para ilustrar que, en el contexto de un carácter mágico, el término pleiotropía se utiliza en un sentido amplio, no sólo aplicable a rasgos fenotípicos distintos sino también a los efectos de un rasgo tanto en la selección como en los cruzamientos (Servedio et al. 2011; pero ver Haller et al. 2012).

Para que la selección divergente pueda operar, es necesario que el polimorfismo del color floral sea temporalmente estable (Gray & McKinnon 2006; Narbona et al. 2018). Su mantenimiento puede deberse a factores bióticos y abióticos, directos e indirectos, si estos ejercen presiones selectivas antagonistas sobre los diferentes morfotipos de color (Rausher 2008; Strauss & Whittall 2006). El color floral es un carácter importante en la atracción a los polinizadores, cuyas fluctuaciones espacio-temporales o cambios

de preferencias pueden originar selección divergente y ser responsables del mantenimiento del polimorfismo (Subramanian & Rausher 2000; Gigord et al. 2001; Smithson 2001; ver revisión en Tremblay et al. 2004). Cuando los polinizadores muestran preferencia por un morfotipo aumentan el flujo génico entre plantas de dicho morfotipo (*assortative mating*) (Hopkins & Rausher 2012; Waser 1993). A menudo los distintos morfotipos de color presentan diferencias en otros caracteres correlacionados, de manera que la selección actúa sobre esos otros caracteres manteniéndose el polimorfismo de color de manera indirecta. Es frecuente que los distintos morfotipos de color estén asociados a diferencias en supervivencia de las plántulas, producción de flores y semillas y resistencia a patógenos y herbívoros (Gigord et al. 2001; Gómez 2000; Carlson & Holsinger 2010). Estas diferencias pueden conferir un *fitness* diferencial a los fenotipos cuando se desarrollan en ambientes heterogéneos, manteniendo el polimorfismo y originando patrones geográficos de distribución de los mismos (Arista et al. 2013; Carlson & Holsinger 2010; Schemske & Bierzychudek 2007). El polimorfismo del color es un precursor de la variación geográfica por lo que puede promover directamente la especiación simpátrica e indirectamente la alopátrica (Forsman et al. 2008).

Hay que considerar que en algunas ocasiones el polimorfismo del color floral no es el carácter que inicia el proceso de especiación, sino que surge de manera secundaria como mecanismo de refuerzo para reducir el coste reproductivo de la interacción entre especies (Pfennig & Pfennig 2010; McEwen & Vamosi 2010; Hopkins 2013; Grossenbacher & Stanton 2014). La evolución del polimorfismo del color como mecanismo de refuerzo ha sido estudiada principalmente en animales (Kirkpatrick & Servedio 1999; Nosil & Yukilevich 2008; Ortiz-Barrientos et al. 2009; Matute 2010), y en menor medida en plantas (Hopkins 2013; Levin & Kerster 1967; Levin 1985; Norton et al. 2015). No obstante, en los últimos años se han hecho importantes avances en el conocimiento de los genes que determinan el polimorfismo del color floral y que están relacionados con el refuerzo (Hopkins & Rausher 2012).

La especiación como proceso continuo

El proceso de especiación no tiene que ser categórico, sino que puede tener lugar de forma progresiva, de manera que el grado de divergencia puede variar cuantitativamente y de la misma manera varía el grado de aislamiento reproductivo, el grado de agrupamiento genotípico, la nitidez de las clinas geográficas en las frecuencias génicas y el grado de agrupación de los linajes (Nosil et al. 2009; Faria et al. 2014). De hecho, existe abundante variabilidad en el estado de especiación, tanto en casos de especiación con flujo génico, como en aquellos en los que hay divergencia en alopatría (Faria et al. 2014; Martin et al. 2013; Zhao et al. 2014). Por

tanto, existe una gradación en el proceso de divergencia de las especies, pudiendo considerarse "etapas de especiación" (Nosil et al. 2009; Fig. 1) que van desde la variación fenotípica incipiente con escaso aislamiento reproductivo, hasta el proceso de especiación completo con diferenciación fenotípica y genotípica de poblaciones y aislamiento reproductivo absoluto (Jiggins & Mallet 2000; Schluter, 2000; Rundle & Nosil, 2005).

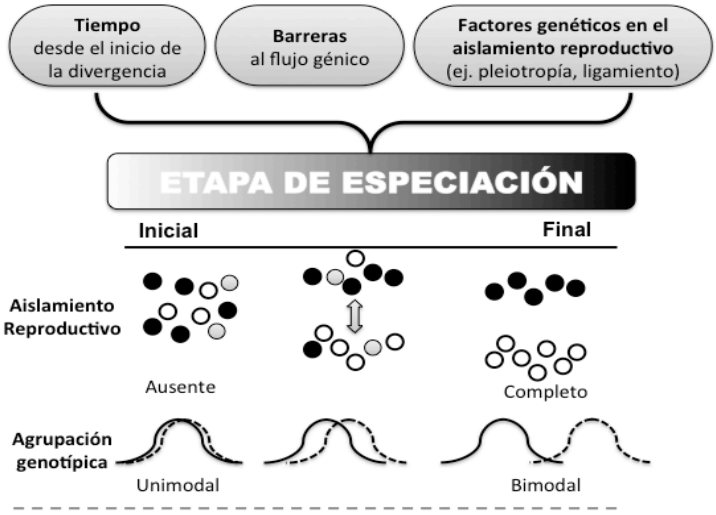

Figura 1. Etapas en el proceso de variación continua de la divergencia durante la especiación, con la situación hipotética de las tres especies de estudio (modificado de Nossil et al., 2009).

Bases moleculares del polimorfismo del color floral: la ruta biosintética de las antocianinas

Los principales grupos de pigmentos que proporcionan el color a los pétalos son flavonoides, betalaínas y carotenoides. Las antocianinas pertenecen a los flavonoides, y son el grupo más diverso (>500 compuestos) y ampliamente representado en las angiospermas (Campanella et al. 2014). En las flores, las antocianinas son las responsables de los colores rojizos, naranjas, rosados, azules y violetas (Miller et al. 2011). La ruta biosintética de las antocianinas (*Anthocyanin Biosynthetic Pathway*, ABP; Fig. 2) es posiblemente una de las mejor conocidas en plantas (Mouradov & Spangenberg 2014). Los genes codificantes de las enzimas y sus respectivos reguladores están secuenciados para una gran cantidad de especies de familias muy diversas (pej. Yuan et al. 2014; Wei et al. 2015; ver revisión en Davies et al. 2012). La ruta posee tres ramas principales que llevan a la síntesis de diferentes antocianinas como productos finales y por tanto el color depende de qué ramas son funcionales. Las ramas principales presentan bifurcaciones laterales que llevan a la síntesis de diferentes tipos de flavonoides que proporcionan resistencia al estrés (Winkel-Shirley 2002; Falcone Ferreyra et al. 2012). La regulación de esta ruta está mediada por el

complejo regulador MBW, un factor de transcripción compuesto por las familias de proteínas R2R3-MYB (MYB), bHLH y WDR (Davies et al. 2012).

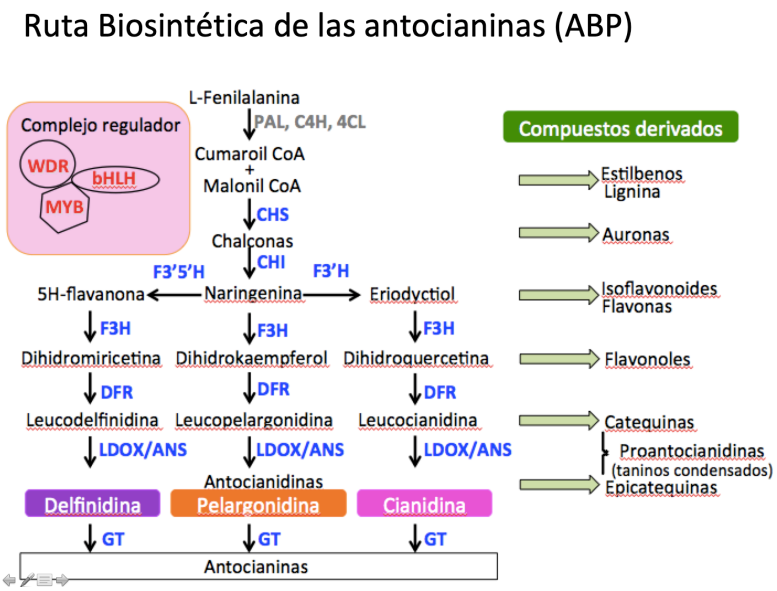

Figura 2. Esquema de la ruta biosintética de las antocianinas en el que se muestran las enzimas principales (azul), las enzimas de la ruta superior de los fenilpropanoides (gris), los compuestos producidos por éstas (negro) y las proteínas reguladoras de los genes que codifican a las enzimas (rojo). Las flechas verdes indican ramas que mediante otras reacciones enzimáticas darán lugar a distintos compuestos secundarios. El cuadro superior rosa muestra los factores reguladores de la transcripción que forman un complejo que controla la síntesis de una o varias proteínas de la ruta. Modificado de Narbona et al. (2014).

El polimorfismo de color floral se debe frecuentemente a mutaciones reguladoras de los factores de transcripción tipo MYB (Wessinger & Rausher 2012; Sobel & Streisfeld 2013), que se expresan a nivel de tejidos o incluso de células, por lo que la ruta puede estar activada en tejidos vegetativos e inactivada en tejidos florales (Petroni & Tonelli, 2011). El aislamiento de los *loci* reguladores del color floral y el análisis de las variaciones moleculares que han tenido lugar en ellos, proporciona una información fundamental sobre el papel del polimorfismo del color en la divergencia de las especies y permite conocer si el polimorfismo del color ha desencadenado un proceso de especiación, o si ha surgido posteriormente como mecanismo de refuerzo (Quattrocchio et al. 1999; Smith et al. 2012).

El objetivo básico de esta tesis doctoral es conocer si el polimorfismo de color floral puede ser un desencadenante de un proceso de especiación en *Lysimachia arvensis* (L.) U. Manns & Anderb. Esta especie estuvo bajo el nombre de *Anagallis arvensis* L. En 2005 un estudio de la filogenia de un grupo de especies de los géneros *Lysimachia* y *Anagallis*, encontró que varias especies de *Anagallis* quedaban situadas dentro del clado formado por las especies de *Lysimachia* (Manns & Anderberg 2005). Tras este

estudio, Manns & Anderberg (2009) propusieron nuevas combinaciones para varias especies de *Anagallis*, entre ellas *A. arvensis*, que pasó a nombrarse *Lysimachia arvensis* (L.) U. Manns & Anderb. *Lysimachia arvensis* es una planta anual de origen mediterráneo y europeo, que ha sido introducida en gran parte del mundo. Tiene dos morfotipos de color: azul y rojo, que se distribuyen en poblaciones puras y mixtas. Estos morfotipos muestran un patrón geográfico de distribución, ya que el rojo está mejor representado en zonas atlánticas y el azul en mediterráneas. Este patrón está asociado a factores abióticos, siendo el morfotipo azul más resistente al estrés ambiental y mejor representado en condiciones áridas (Arista et al. 2013). En invernadero, los morfos presentaron un ligero desfase en la fenología de la floración, lo que sugiere que la mutación que ha causado el cambio en el color floral podría haber afectado pleiotrópicamente al momento de floración (Arista et al. 2013). En el Mediterráneo, los polinizadores prefieren el morfotipo azul, sobre el que ejercen selección direccional positiva, se desconoce qué mecanismo es el responsable del mantenimiento del morfotipo rojo, aunque se ha propuesto como posibilidad su capacidad de autopolinizarse (Ortiz et al. 2015). Sin embargo, la especie presenta hercogamia, lo que podría dificultar la deposición de polen propio. El patrón geográfico encontrado, el ligero desfase fenológico y las preferencias de los polinizadores sugieren que podría existir una mayor frecuencia de cruzamientos entre los morfotipos de un mismo color (*assortative mating*) lo que originaría aislamiento reproductivo y abriría las puertas a un proceso de especiación, sentando las bases de esta tesis.

OBJETIVOS, ESTRUCTURA Y PRINCIPALES RESULTADOS DE LA TESIS DOCTORAL

Esta tesis persigue los siguientes objetivos específicos:

1-Conocer cómo se segrega el color floral.

2-Conocer si los morfotipos de color difieren en su sistema reproductivo y si presentan depresión por endogamia cuando se reproducen por autogamia.

3-Conocer la diversidad genética de las poblaciones de ambos morfos de color

4-Describir la hercogamia en la especie y conocer su heredabilidad.

5-Determinar si la hercogamia evita la autopolinización automática en cada uno de los dos morfotipos de color y conocer la variabilidad de este carácter en poblaciones mixtas y puras.

6-Establecer la presencia de barreras reproductivas precigóticas y postcigóticas entre los dos morfotipos de color.

7-Conocer dónde se sitúan los dos morfotipos de color en la filogenia del grupo de especies Mediterráneas con las que *L. arvensis* está emparentada.

Para responder a esos objetivos, la tesis doctoral se encuentra estructurada en nueve capítulos, dos de los cuales, Introducción (1) y Discusión General (9) introducen el problema de estudio y discuten los resultados obtenidos a lo largo de la tesis. Los resultados más importantes de los capítulos 2 al 8 se describen a continuación.

En el **Capítulo 2** se aborda la segregación del color floral y se trata de determinar si los polinizadores, básicamente abejas, son capaces de diferenciar los distintos morfotipos de color que surgen del cruce entre el rojo y azul. Hemos encontrado que el cruce entre morfos distintos origina una F1 homogénea de color salmón cuyo cruce con los parentales rojo o azul origina una F2 donde vuelven a aparecer plantas con flores azules, rojas y salmones, pero también una pléyade de estadios de color intermedios entre el rojo y el salmón. Cuando el espectro de reflectancia de estos morfos de color se representa en el hexágono de color de Chittka, encontramos que el azul es el que contrasta más con el fondo verde de las hojas, por lo que debe ser el más fácilmente encontrado por las abejas. El morfo salmón y el rojo se diferencian entre sí muy poco por lo que cabe pensar que no serían distinguibles por las abejas en base a su color. Los resultados de este capítulo se han enviado para su publicación a la revista Plant Biosystems.

En el **Capítulo 3**, se describe el diseño de 12 *loci* de microsatélites para *Lysimachia arvensis* como punto de partida para el estudio posterior de la diversidad genética de los moros. La variabilidad de estos *loci* se ha probado en el morfo azul y el rojo y también en *Lysimachia monelli*, una especie hermana de *L*. *arvensis*. Como resultado se han obtenido marcadores microsatélites con variabilidad que pueden ser aplicados a estudios de genética de poblaciones y que se ha probado que funcionan en *L*. *monelli*. Los resultados de este capítulo han sido publicados en la revista Bichemical, Systematic & Ecology.

En el **Capítulo 4**, se estudia la expresión de la depresión por endogamia de los dos morfos de color a lo largo del ciclo reproductivo completo en dos poblaciones naturales mediterráneas. Además, se estudia la diversidad genética de ambos morfos en 20 poblaciones Mediterráneas y no Mediterráneas mediante el uso de marcadores AFLP y microsatélites. Hemos encontrado que los dos morfos mostraron depresión por endogamia en casi todas las etapas de su ciclo de vida, pero en diferente magnitud. El morfo azul mostró una depresión por endogamia acumulada de entre 0,18 y 0,32, mientras que la del morfo rojo fue muy superior de entre 0,61 y 0,65. Los morfos mostraron además diferencias en la fenología de la germinación y la floración en función del origen autógamo o xenógamo de sus semillas. El morfo azul y el rojo

presentaron una enorme diferenciación genética entre ellos y la diversidad genética del morfo rojo fue muy inferior a la del azul tanto en poblaciones Mediterráneas como no Mediterráneas. Los resultados de este capítulo sugieren que los dos morfos son actualmente linajes bien diferenciados, por lo que a partir de este capítulo nos referiremos a ellos como linajes. Los resultados de este capítulo se han enviado a una revista para su revisión.

En el **Capítulo 5** se describirá la presencia de dos tipos consecutivos de hercogamia, lateral y vertical, que aparecen en las flores de *L. arvensis*. Se ha encontrado que en el primer día de antesis las flores muestran hercogamia lateral en la que el estilo forma un ángulo de hasta 60 grados con los estambres. En el segundo día, el estilo se mueve y se coloca entre los estambres pudiendo quedar el estigma por encima de las anteras (hercogamia de aproximación), por debajo (hercogamia reversa) o al mismo nivel (no hercogamia). Ambos tipos de hercogamia muestran muy poca variabilidad intraplanta y un alto grado de heredabilidad, de h^2=0.843 para la lateral y de h^2=0.635 para la vertical. Los resultados de este capítulo han sido publicados en la revista Plant Species Biology.

En el **Capítulo 6** se estudia la relación entre el nivel de hercogamia y la autodeposición del polen en ausencia de polinizadores y se estudia la distribución de este carácter entre linajes y en función de si viven juntos o separados. La hercogamia lateral fue la más eficiente controlando la auto-deposición de polen propio, ya que por encima de 20 grados prácticamente no se produce autopolinización automática. En la hercogamia vertical, solo la de aproximación fue eficiente evitando la autopolinización. Los linajes de color mostraron diferencias muy consistentes en la expresión de la hercogamia. Las flores del linaje rojo mostraron una marcada hercogamia lateral en el primer día y hercogamia reversa durante el segundo día, configuración que favorece el "delayed selfing". El azul mostró mucha más variabilidad, con poblaciones con marcada hercogamia lateral y hercogamia de aproximación, y por tanto xenógamas, y otras con poca hercogamia lateral y hercogamia de aproximación, lo que favorece el "competing selfing". Ambos linajes mostraron una significativa reducción de la hercogamia en poblaciones mixtas, lo que sugiere una evolución hacia la autogamia en esas poblaciones. Los resultados de este capítulo se han enviado a una revista para su revisión.

El Capítulo 7, es el capítulo central de esta tesis ya que en él se estudian cada una de las barreras pre- y postcigóticas que pueden aparecer entre los dos linajes de color y que determinan el grado final de aislamiento reproductivo. Entre las barreras precigóticas se han estudiado las geográficas, las fenológicas, las dirigidas por los polinizadores y la precedencia del polen. Entre las postcigóticas se han estudiado las

relacionadas con el cruce entre morfos: formación de frutos y semillas, germinación, viabilidad y supervivencia de las plántulas, producción de frutos y esterilidad de la F2. Hemos encontrado que las barreras precigóticas son mucho más importantes que las postcigóticas. El aislamiento geográfico, la precedencia del polen y el aislamiento por polinizadores fueron las barreras más importantes. El índice de aislamiento global entre linajes fue de un 0,7855. Los resultados de este capítulo se están elaborando para su publicación.

El Capítulo 8 aborda mediante el uso de marcadores ITS y cloroplásticos la filogenia del clado de especies de *Lysimachia* en el que se encuentra *L. arvensis,* teniendo en cuenta los linajes de color en *L. arvensis* y los dos morfos de color, azul y rojo, que aparecen también en *L. monelli*. Además, se incluyen las especies *L. foemina*, y *L. talaverae* ambas de flores azules. Hemos encontrado que los 4 marcadores cloroplásticos utilizados agrupan los individuos de *L. arvensis* con independencia de su color floral y lo mismo ocurre en el caso de *L. monelli*. Sin embargo, los marcadores ITS agrupan las muestras del linaje rojo de *L. arvensis* con los dos morfos de *L. monelli* y con *L. foemina*. En un clado diferente queda el linaje azul de *L. arvensis* junto con *L. talaverae*. Los resultados sugieren que tanto *L. arvensis* como *L. monelli* están compuestas por dos linajes totalmente diferentes que pueden ser considerados bajo la categoría de especies, por lo que se propone una nueva combinación para estos taxones. Los resultados de este capítulo están terminando de elaborarse para ser enviados a publicar.

REFERENCIAS

Arista M., Talavera M., Berjano R. & Ortiz P.L. (2013). Abiotic factors may explain the geographical distribution of flower colour morphs and the maintenance of colour polymorphism in the scarlet pimpernel. *Journal of Ecology*, **101***:* 1613-1622.

Bradshaw H.D. & Schemske D.W. (2003). Allele substitution at a flower colour *locus* produces a pollinator shift in monkeyflowers. *Nature*, **426**: 176.

Butlin R. & Ritchie M.G. (2001). Evolutionary biology: searching for speciation genes. *Nature*, **412**: 31.

Campanella J.J., Smalley J.V. & Dempsey M.E. (2014). A phylogenetic examination of the primary anthocyanin production pathway of the Plantae. *Botanical studies*, **55**: 10.

Carlson J.E. & Holsinger K.E. (2010). Natural selection on inflorescence color polymorphisms in wild Protea populations: the role of pollinators, seed predators, and intertrait correlations. *American Journal of Botany*, **97**: 934-944.

Carlson J.E. & Holsinger K.E. (2013). Direct and indirect selection on floral pigmentation by pollinators and seed predators in a color polymorphic South African shrub. *Oecologia*, **171**: 905-919.

Coyne J.A. & Orr H.A. (2004). Speciation. Sinauer Associates. Sunderland, MA.

Coyne J.A. (2007). Sympatric speciation. *Current Biology*, **17**: R787-R788.

Davies K.M., Albert N.W. & Schwinn K.E. (2012). From landing lights to mimicry: the molecular regulation of flower colouration and mechanisms for pigmentation patterning. Functional Plant Biology, **39**: 619-638.

Dobzhansky T. (1937). *Genetics and the Origin of Species*. Columbia Univ. Press.

Falcone Ferreyra M.L., Rius S. & Casati P. (2012). Flavonoids: biosynthesis, biological functions, and biotechnological applications. Frontiers in plant science, **3**: 222.

Faria R., Renaut S., Galindo J., Pinho C., Melo-Ferreira J., Melo M., Jones F., Salzburger W., Schluter D. & Butlin R. (2014). Advances in Ecological Speciation: an integrative approach. *Molecular ecology*, **23**: 513-521.

Forsman A., Ahnesjö J., Caesar S. & Karlsson M. (2008). A model of ecological and evolutionary consequences of color polymorphism. *Ecology*, **89**: 34-40.

Funk D.J., Nosil P., & Etges W.J. (2006). Ecological divergence exhibits consistently positive associations with reproductive isolation across disparate taxa. *Proceedings of the National Academy of Sciences*, **103**: 3209-3213.

Gigord L.D., Macnair M.R. & Smithson A. (2001). Negative frequency-dependent selection maintains a dramatic flower color polymorphism in the rewardless orchid *Dactylorhiza sambucina* (L.) Soo. *Proceedings of the National Academy of Sciences*, **98**: 6253-6255.

Gómez J.M. (2000). Phenotypic selection and response to selection in *Lobularia maritima*: importance of direct and correlational components of natural selection. *Journal of Evolutionary Biology*, **13**: 689-699.

Gray S.M. & McKinnon J.S. (2007). Linking color polymorphism maintenance and speciation. *Trends in ecology & evolution*, **22**: 71-79.

Grossenbacher D.L. & Stanton M.L. (2014). Pollinator-mediated competition influences selection for flower-color displacement in sympatric monkeyflowers. *American journal of botany*, **101**: 1915-1924.

Haller **B.C., De Léon L.F., Rolshausen G., Gotanda K.M. & Hendry A.P.** (2012). Magic traits: distinguishing the important from the trivial. *Trends in ecology & evolution*, **27**: 4-5.

Hopkins R. & Rausher M.D. (2012). Pollinator-mediated selection on flower color allele drives reinforcement. *Science*, **335**: 1090-1092.

Hopkins R. (2013). Reinforcement in plants. *New Phytologist*, **197**: 1095-1103.

Hugall A.F. & Stuart-Fox D. (2012). Accelerated speciation in colour-polymorphic birds. *Nature*, **485**: 631.

Huxley J. (1955). Morphism and evolution. *Heredity*, **9**: 1.

Imbert E., Wang H., Anderson B., Hervouet B., Talavera M. & Schatz B. (2014). Reproductive biology and colour polymorphism in the food-deceptive *Iris lutescens* (Iridaceae). *Acta Botanica Gallica*, **161**: 117-127.

Irwin R.E., Strauss S.Y., Storz S., Emerson A. & Guibert G. (2003). The role of herbivores in the maintenance of a flower color polymorphism in wild radish. *Ecology*, **84**: 1733-1743.

Jiggins C.D. & Mallet J. (2000). Bimodal hybrid zones and speciation. *Trends in ecology & evolution*, **15**: 250-255.

Kay K.M. (2006). Reproductive isolation between two closely related hummingbird pollinated neotropical gingers. *Evolution*, **60**: 538-552.

Kay K.M. & Sargent R.D. (2009). The role of animal pollination in plant speciation: integrating ecology, geography, and genetics. *Annual Review of Ecology, Evolution, and Systematics*, **40**: 637-656.

Kirkpatrick M. & Servedio M.R. (1999). The reinforcement of mating preferences on an island. *Genetics*, **151**: 865-884.

Levin D.A. (1985). Reproductive character displacement in Phlox. *Evolution*, **39**: 1275-1281.

Levin D.A. (2010). Environment-enhanced self-fertilization: implications for niche shifts in adjacent populations. *Journal of Ecology*, **98**: 1276-1283.

Levin D.A. & Brack E.T. (1995). Natural selection against white petals in Phlox. *Evolution*, **49**: 1017-1022.

Levin D.A. & Kerster H.W. (1967). Natural selection for reproductive isolation in Phlox. *Evolution*, **21**: 679-687.

Lowry D.B., Modliszewski J.L., Wright K.M., Wu C.A. & Willis J.H. (2008). The strength and genetic basis of reproductive isolating barriers in flowering plants. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **363**: 3009-3021.

Manns U. & Anderberg A.A. (2005). Molecular phylogeny of *Anagallis* (Myrsinaceae) based on ITS, trn LF, and ndh F sequence data. *International journal of plant sciences*, **166**: 1019-1028.

Manns U. & Anderberg A.A. (2009). New combinations and names in *Lysimachia* (Myrsinaceae) for species of *Anagallis*, *Pelletiera* and *Trientalis*. *Willdenowia*, **39**: 49-54.

Martin N.H. & Willis J.H. (2007). Ecological divergence associated with mating system causes nearly complete reproductive isolation between sympatric *Mimulus* species. *Evolution*, **61**: 68-82.

Martin S.H., Dasmahapatra K.K., Nadeau N.J., Salazar C., Walters J.R., Simpson F., Blaxter M., Manica A., Mallet J. & Jiggins C.D. (2013). Genome-wide evidence for speciation with gene flow in *Heliconius* butterflies. *Genome research*, gr-159426.

Matute D.R. (2010). Reinforcement can overcome gene flow during speciation in *Drosophila*. *Current Biology*, **20**: 2229-2233.

Mayr E. (1942). *Systematics and the origin of species from the viewpoint of a zoologist*. Harvard University Press.

Mayr E. (1963). *Animal species and evolution*. Harvard University Press.

McEwen J.R. & Vamosi J.C. (2010). Floral colour versus phylogeny in structuring subalpine flowering communities. *Proceedings of the Royal Society of London B: Biological Sciences*, rspb20100501.

McLean C.A. & Stuart-Fox D. (2014). Geographic variation in animal colour polymorphisms and its role in speciation. *Biological Reviews,* **89**: 860-873.

Miller R., Owens S.J. & Rørslett B. (2011). Plants and colour: flowers and pollination. *Optics & Laser Technology*, **43**: 282-294.

Mouradov A. & Spangenberg G. (2014). Flavonoids: a metabolic network mediating plants adaptation to their real estate. *Frontiers in plant science, **5**,* 620.

Muller H. (1942). Isolating mechanisms, evolution, and temperature. *Biology Symposium,* **6**: 71-125.

Narbona E., Buide M.L., Casimiro-Soriguer I. & Del Valle J.C. (2014). Polimorfismos de color floral: causas e implicaciones evolutivas. *Ecosistemas*, **23**: 36-47.

Narbona E., Wang H., Ortiz P.L., Arista M., & Imbert E. (2018). Flower colour polymorphism in the Mediterranean Basin: Occurrence, maintenance and implications for speciation. *Plant Biology*, **20**: 8-20.

Noor M.A. (2003). Evolutionary biology: genes to make new species. *Nature*, **423**: 699.

Norton N.A., Fernando M.T.R., Herlihy C.R. & Busch J.W. (2015). Reproductive character displacement shapes a spatially structured petal color polymorphism in *Leavenworthia stylosa*. *Evolution*, **69**: 1191-1207.

Nosil P. & Yukilevich R. (2008). Mechanisms of reinforcement in natural and simulated polymorphic populations. *Biological Journal of the Linnean Society*, **95**: 305-319.

Nosil P., Harmon L.J. & Seehausen O. (2009). Ecological explanations for (incomplete) speciation. *Trends in ecology & evolution*, **24**: 145-156.

Orr H.A., Masly J.P. & Phadnis N. (2006). Speciation in *Drosophila*: from phenotypes to molecules. *Journal of Heredity*, **98**: 103-110.

Ortiz P.L., Berjano R., Talavera M., Rodríguez-Zayas L. & Arista M. (2015). Flower colour polymorphism in *Lysimachia arvensis*: How is the red morph maintained in Mediterranean environments?. *Perspectives in Plant Ecology, Evolution and Systematics*, **17**: 142-150.

Ortiz-Barrientos D., Grealy A. & Nosil P. (2009). The genetics and ecology of reinforcement: implications for the evolution of prezygotic isolation in sympatry and beyond. *Annals of the New York Academy of Sciences*, **1168**: 156-182.

Petroni K. & Tonelli C. (2011). Recent advances on the regulation of anthocyanin synthesis in reproductive organs. *Plant science*, **181**: 219-229.

Pfennig D.W. & Pfennig K.S. (2010). Character displacement and the origins of diversity. *The american naturalist*, **176**: S26-S44.

Presgraves D.C. (2010). The molecular evolutionary basis of species formation. *Nature Reviews Genetics*, **11**: 175.

Quattrocchio F., Wing J., van der Woude K., Souer E., de Vetten N., Mol J. & Koes R. (1999). Molecular analysis of the anthocyanin gene of petunia and its role in the evolution of flower color. *The Plant Cell*, **11**: 1433-1444.

Rausher M.D. (2008). Evolutionary transitions in floral color. *International Journal of Plant Sciences*, **169**: 7-21.

Rieseberg L.H. & Blackman B.K. (2010). Speciation genes in plants. *Annals of Botany*, **106**: 439-455.

Rieseberg L.H. & Willis J.H. (2007). Plant speciation. *science*, **317**: 910-914.

Rieseberg L.H., Church S.A. & Morjan C.L. (2004). Integration of populations and differentiation of species. *New Phytologist*, **161**: 59-69.

Rosenblum E.B. (2005). Convergent evolution and divergent selection: lizards at the White Sands ecotone. *The American Naturalist*, **167**: 1-15.

Rundle H.D. & Nosil P. (2005). Ecological speciation. *Ecology letters*, **8**: 336-352.

Rundle H.D., Nagel L., Boughman J.W. & Schluter D. (2000). Natural selection and parallel speciation in sympatric sticklebacks. *Science*, **287**: 306-308.

Runquist R.D. & Moeller D.A. (2013). Floral and mating system divergence in secondary sympatry: testing an alternative hypothesis to reinforcement in *Clarkia*. *Annals of botany*, **113**: 223-235.

Schemske D.W. & Bierzychudek P. (2007). Spatial differentiation for flower color in the desert annual *Linanthus parryae*: was Wright right?. *Evolution: International Journal of Organic Evolution*, **61**: 2528-2543.

Schluter D. (2000). *The Ecology of Adaptive Radiation*. Oxford University Press. Oxford.

Servedio M.R., Van Doorn G.S., Kopp M., Frame A.M. & Nosil P. (2011). Magic traits in speciation: 'magic' but not rare?. *Trends in ecology & evolution*, **26**: 389-397.

Smith S.D., Wang S. & Rausher M.D. (2012). Functional evolution of an anthocyanin pathway enzyme during a flower color transition. *Molecular biology and evolution*, **30**: 602-612.

Smithson A. (2001). Pollinator preference, frequency dependence, and floral evolution. 237-257. In: Chittka L., Thomson J.H. (eds). *Cognitive ecology of pollination*. Cambridge University Press.

Sobel J.M. & Chen G.F. (2014). Unification of methods for estimating the strength of reproductive isolation. *Evolution*, **68**: 1511-1522.

Sobel J.M. & Streisfeld M.A. (2013). Flower color as a model system for studies of plant evo-devo. *Frontiers in plant science*, **4**: 321.

Sobel J.M., Chen G.F., Watt L.R. & Schemske D.W. (2010). The biology of speciation. *Evolution*, **64**: 295-315.

Strauss S.Y. & Whittall J.B. (2006). *Non-pollinator agents of selection on floral traits*, 120-138. In: Harder L.D. & Barrett S.C.H. (eds). *Ecology and evolution of flowers*. Oxford University Press.

Subramaniam B. & Rausher M.D. (2000). Balancing selection on a floral polymorphism. *Evolution*, **54**: 691-695.

Tremblay R.L., Ackerman J.D., Zimmerman J.K. & Calvo R.N. (2004). Variation in sexual reproduction in orchids and its evolutionary consequences: a spasmodic journey to diversification. *Biological Journal of the Linnean Society*, **84**: 1-54.

Vercken E. & Clobert J. (2008). The role of colour polymorphism in social encounters among female common lizards. *The Herpetological Journal*, **18**: 223-230.

Via S. (2009). Natural selection in action during speciation. *Proceedings of the National Academy of Sciences*, **106**: 9939-9946.

Waser N.M. (1986). Flower constancy: definition, cause, and measurement. *The American Naturalist*, **127**: 593-603.

Waser N.M. (1993). Population structure, optimal outbreeding, and assortative mating in angiosperms. 173-199. In: Thornhill W.N. *The natural history of inbreeding and outbreeding*, University of Chicago Press.

Wei C., Tao X., Li M., He B., Yan L., Tan X., & Zhang Y. (2015). De novo transcriptome assembly of Ipomoea nil using Illumina sequencing for gene discovery and SSR marker identification. *Molecular genetics and genomics*, **290**: 1873-1884.

Wessinger C.A. & Rausher M.D. (2014). Ecological transition predictably associated with gene degeneration. *Molecular biology and evolution*, **32**: 347-354.

Wheldale M. (1916). *Anthocyanic pigments of plants.* Cambridge University Press.

Whittall J.B., Syring J., Parks M., Buenrostro J., Dick C., Liston A. & Cronn R. (2010). Finding a (pine) needle in a haystack: chloroplast genome sequence divergence in rare and widespread pines. *Molecular Ecology*, **19**: 100-114.

Widmer A., Lexer C. & Cozzolino S. (2009). Evolution of reproductive isolation in plants. *Heredity*, **102**: 31.

Winkel-Shirley B. (2002). Biosynthesis of flavonoids and effects of stress. *Current opinion in plant biology*, **5**: 218-223.

Wright S. (1978). *Evolution and the genetics of population, variability within and among natural populations*. The University of Chicago Press.

Wu C.A., Streisfeld M.A., Nutter L.I. & Cross K.A. (2013). The genetic basis of a rare flower color polymorphism in *Mimulus lewisii* provides insight into the repeatability of evolution. *PLoS One*, 8: e81173.

Yuan Y.W., Sagawa J.M., Frost L., Vela J.P. & Bradshaw H.D. (2014). Transcriptional control of floral anthocyanin pigmentation in monkeyflowers (*Mimulus*). *New Phytologist*, **204**: 1013-1027.

Zhao W., Meng J., Wang B., Zhang L., Xu Y., Zeng Q.Y., Li Y., Mao J.M. & Wang X.R. (2014). Weak crossability barrier but strong juvenile selection supports ecological speciation of the hybrid pine *Pinus densata* on the Tibetan plateau. *Evolution*, **68**: 3120-3133.

2. Flower colour segregation and flower characterization under the bee vision model in the polymorphic *Lysimachia arvensis*.

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ABSTRACT

Floral colour determines pollinator behaviour and thus strongly affecting plant-mating systems. *Lysimachia arvensis* is a polymorphic species with blue- and red-flowered plants, which colour inheritance remains largely unknown. A control of floral colour based on one locus, with the red allele as dominant, has been proposed. This proposal cannot explain the sporadic appearance of other floral colours in wild populations, perhaps due to spontaneous mutations. We studied floral colour segregation in *L. arvensis* and assessed the possibility that pollinators can visually distinguish colour morphs. Hand crossing between both morphs originated a homogeneous F1 with salmon-coloured flowers. In the F2, blue, red, salmon morphs and other plants with intermediate colours appeared, suggesting that more than one single locus is involved in colour segregation. When flower reflectance spectra are plotted on bee colour space of Chittka, their positions suggest that bees are not able to distinguish between red and salmon flowers. Moreover, pollinators would perceive more easily the blue flowers, due to their higher contrast to the background. Our study shows that "flower colour" is a natural marker to assess the rate of crossing between morphs. The extreme rarity of salmon flowers in wild populations of *L. arversis* indicates assortative mating.

Key words: *Anagallis*, Chittka hexagon, floral evolution, flower colour quantification, pollinator preference

INTRODUCTION

Angiosperms exhibit a markedly high diversity of flower colours, with sister species usually differing in intensity, hue or colour pattern of the corolla (eg., Rausher 2008; Smith et al. 2010; Smith and Rausher 2011; Lagomarsino et al. 2017). This diversity implies that there have been numerous evolutionary transitions in the colour of flowers (Weis 1995; Rausher 2008). Flower colour is often correlated with other floral traits, resulting in the common recognition of "pollination syndromes" (Fenster et al. 2004). Flower colour has an enormous importance as a claim in the attraction of pollinators that may have preferences for some colours over others (Chittka and Menzel 1992), so that transitions to different colours may represent adaptation to different sets of pollinators (Faegri and van der Pijl 1966; Grant 1993; Fenster et al. 2004; Rausher 2008). In general, the colour of the flowers is due to the presence of pigments (Kay et al. 1981; Van der koi et al. 2016). There are four large groups of pigments: chlorophylls, carotenoids, betalains and flavonoids. Among these, anthocyanins, a group of flavonoids, are the most important floral pigments and are produced in a well-known and conserved biosynthetic pathway in angiosperms (Rausher et al. 1999).

Flower colour polymorphism is the presence of more than one colour morph, genetically determined, within the populations of a species (Huxley 1955). This phenomenon appears by a spontaneous mutation in the biosynthetic route of the pigments that give colour to the flowers. Once a coloured mutant appears in a population, this recent polymorphism can be lost or maintained depending on biotic or abiotic selective factors and gene drift (Narbona et al. 2018). Pollinator preferences play a fundamental role in the maintenance or loss of the flower colour polymosphism. The behavior of pollinators can induce changes in plant fertility, cross-pollination ratios, and pollen flow among colour morphs (Malerba and Nattero 2012). These changes can lead to the genetic differentiation of individuals with different flower colour, promoting ultimately the speciation processes (Servedio et al. 2011). Obviously, in order for pollinators to discriminate between floral colours and act as selection agents, it is imperative they can differentiate them visually. Therefore, a subjective evaluation of the floral colours according to the human vision can lead to missleading interpretations in relation to the behavior of pollinators, being necessary an objective measurements of colours and their evaluation according to the visual system of pollinators.

Lysimachia arvensis (L.) U. Manns & Anderb. is a tetraploid annual species, native to the Mediterranean Basin and Europe that presents flower colour polymorphism. In natural populations there are plants with blue and red flowers, and these colours are due to the presence of different types of anthocyanins. Malvidin is mainly responsible for the blue colour and pelargonidin for the red colour (Wiering and de Vlaming in Harborne 1968; Ishikura 1981). Selective abiotic factors influence a geographic distribution pattern of colour morphs,

with blue being much better represented in more xeric environments (Arista et al. 2013). In addition, in Mediterranean environments pollinators show a higher preference for the blue morph and the red morph has lower fitness; despite this, it still remains in the populations although in a low proportion (Ortiz et al. 2015).

The inheritance of flower colour in *L. arvensis* is unknown, and unravel it could help to understand the maintenance of the red morphotype in Mediterranean populations, despite being subject to negative selection (Arista et al. 2013). In a simple scenario, if a recessive allele were responsible for the red colour, it would be protected in the heterozygotes that would show the blue dominant phenotype. However, in an oral communication in a Congress in 1910, Weiss explained that in experimental crosses between plants with flowers of different colour, the F1 obtained was all homogeneously red. Therefore, he concluded that the flower colour in *L. arvensis* depended on a single gene with two alleles, being the red allele dominant over the blue. The fact that flowers of intermediate colour do not usually appear in natural populations would support this dominance-recessive relationship between both alleles. Later, Marsden-Jones & Weiss (1938) confirmed that result, although in some populations they found some plants of *L. arvensis* with flowers of salmon colour and others of pale blue colour. Salmon flowered plants, although rare, had been also described previously by other authors who had suggested a hybrid origin between blue and red morphs since they only appeared when the two morphs, blue and red, coexist (Hoffmann 1879; Pax 1905). However, Marsden-Jones & Weiss (1938) found these plants in monomorphic red populations, and thus they attributed salmon plants to spontaneous mutations. In a recent sampling over 19 mixed populations of *Lysimachia arvensis* in Western Europe, salmon-flowered plants appeared in two of them (Canarias and Aracena, Jiménez-López et al., unpub results). Their scarce representation in populations makes it difficult to know if they result from spontaneous mutations or by crossing between the red and the blue morphs. In the latter case, only a low frequency of crossing between morphs or a low success of the progeny of that crossing would explain the almost abesnece of salmon-flowered individuals in mixed natural populations.

The objectives of the present work are: (1) to establish if the salmon morph results from the crossing between the blue and the red morph in *Lysimachia arvensis*, (2) to know how the flower colour is inherited, (3) to characterize quantitatively the flower colours that can be appear in this species and (4) to determine if they can be differentiated by pollinators. This last objective has focused specifically on the vision system of bees since they are the main floral visitors of *L. arvensis* (Ortiz et al., 2015).

MATERIAL AND METHODS

Heritability of flower colour

To study the inheritance of colour in L. arvensis, hand pollinations have been carried out in the greenhouse. Flower colour segregation was first quantified in offspring based on human vision. The plants used originally came from seeds obtained in natural populations of Hinojos (Spain), Tangier (Morocco), Tabarka (Tunisia) and Corsica (France). These plants were grown in greenhouse, and by manual self-pollinations two successive generations were obtained to select pure colour lines. These pure lines, blue (B) and red (R) were used as parental (P) in this study. Crossings were carried out between parents of the same colour and different colour in order to obtain the F1. Crosses between parents of different colours were carried out in both directions, that is, the blue plants as pollen donors and the red plants as pollen receiver (RxB, n = 84 crosses) and the red plants as pollen donors and the blue as receiver (BxR, n = 88). The F1 seeds obtained were put to germinate in Petri dishes in germination chambers under 16h of light at 22°C and 8h of darkness at 15°C and seedlings were grown in the greenhouse. In this F1, different types of pollinations were made to obtain the F2. Some F1 plants were self-pollinated (N=149 pollinations), others were crossed each other (N = 43 crosses), others were backcrossed with blue parental (N = 34 crosses), and others with red parental (N = 39 crosses). All seeds produced by this F1 were germinated and the resulting seedlings were grown in greenhouses until flowering (2907 plants).

Flower colour characterization

To characterize quantitatively floral colours of *L. arvensis* plants obtained from the crossing program previously described, the reflectance spectra of the petals of a subsample of plants were measured. The reflectance was measured in 88 parental plants (44 B and 44 R), 38 F1 plants (S thereafter; 15 from BxR and 23 from RxB) and 41 F2 plants obtained from self-pollination of the F1. Reflectance was also measured in 53 plants from the F1 backcrosses with both parents (19 from SxB, 5 from BxS, 14 from SxR and 15 from RxS). In each plant, the reflectance of the adaxial surface of a petal was measured, discarding the basal part corresponding to the centre of the flower (bullseye). To do this, a JAZ A1465 double-beam spectrophotometer from Ocean Optics, equipped with a UV-visible light source and capable of measuring reflectance between 190 and 890 nm was used.

To assess how these petals are perceived by bees, the reflectance values between 300 and 700 nm obtained in each measurement were elaborated and represented in the colour hexagon model. This model was developed by Chittka (1992) integrating experimental data related to the reception of visual signals by bees and the translation of these signals in the bee brain. The colour hexagon is a two-dimensional representation in which each point of reflectance corresponds to a point defined by some Cartesian coordinates; a detailed

description of how to transfer the reflectance data to the colour hexagon can be seen in Chittka & Kevan (2005). This model allows to quantify the colour contrast of colour between two flowers perceived by the bees as the Euclidean distance in the hexagon between the points generated by their colour spectra. It also allows to quantify the contrast of a flower with the green background as the Euclidean distance between the point generated by the flower spectrum and the center of the hexagon. In addition, this model allows the categorization in a conventional manner of the colours perceived by bees placing them in six colour categories. The colour contrast of each flower with the green background was quantified. Differences in colour contrast among parents, F1 and F2 were tested by means of one-way ANOVA followed by a Tukey test. Moreover, the colour contrast of each flower with those of the rest of the flowers sampled was quantified.

RESULTS

Heritability of flower colour

All offspring obtained from crosses BxB and RxR was homogeneous and showed the same colour as the parents (N = 350 individuals observed in each case), which confirms the purity of the blue and red lines selected as parental. The crosses between plants of different colour, BxR and RxB, also originated a homogeneous offspring salmon in colour (N = 1199 individuals analyzed, Fig. 1). In addition, these individuals presented a bullseye (ring of colour at the base of the petals) similar insize than that of the blue morph but larger than that of the red morph (Fig. 1). The self-pollination of the F1 originated 707 blue plants, 926 red and 452 salmon, but also appeared 51 individuals with intermediate colours between red and

Figure 1. Flowers of *Lysimachia arvensis*. A: blue (top left), orange (top right) and salmon F1 (bottom) morphs. B: sample of individuals resulting from the self-pollination of F1 or from the backcrosses of F1 with its parents.

salmon (Figs 1 and 2). The backcrosses of the F1 with each of the parents also gave rise to these four phenotypes, but in different proportions (Fig. 2). When the backcross was performed with the blue parent, offspring showed mainly blue flowers (n = 407 plants), whereas when it was carried out with the red parent offspring was predominatly red (n = 496; Fig. 2).

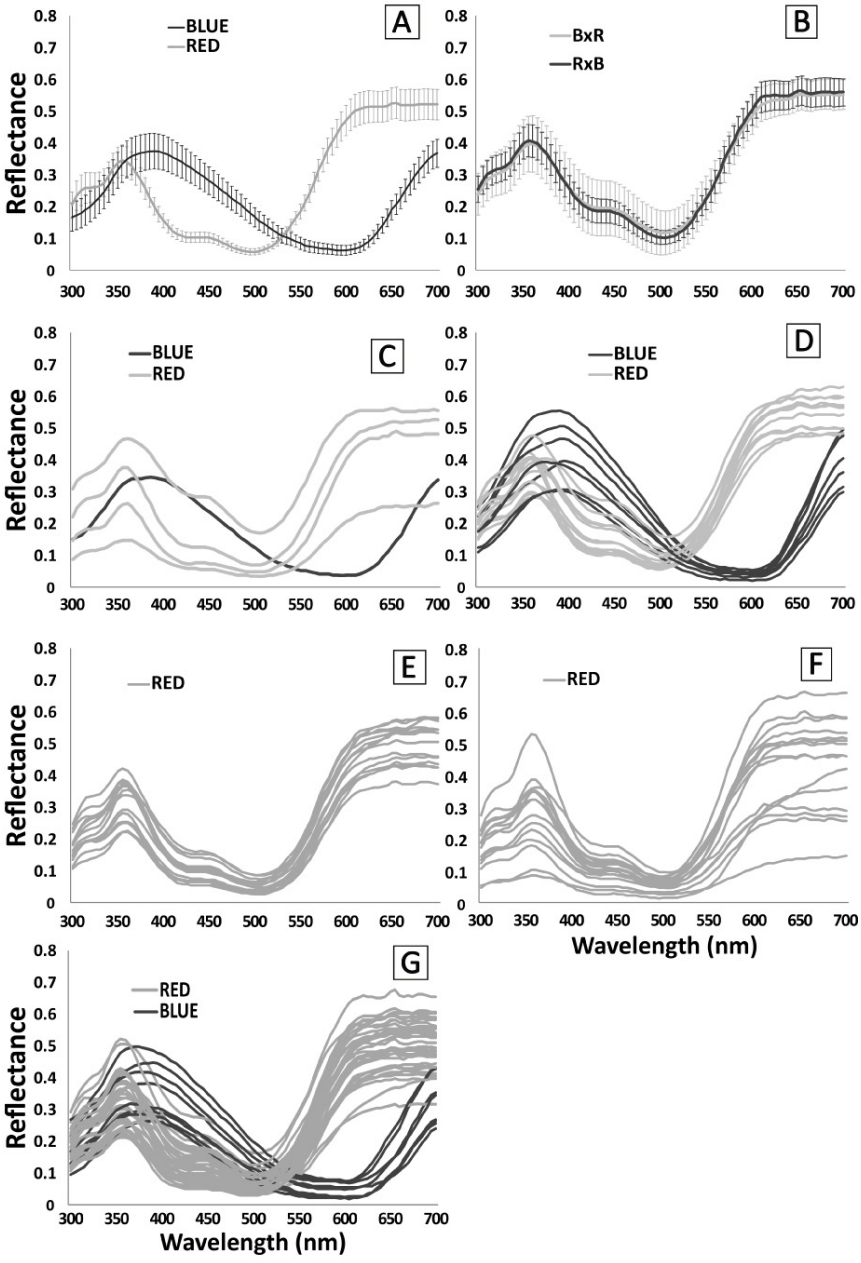

Figure 2. Reflectance spectra of the flower color of *Lysimachia arvensis*. A: blue and red morphs. B: F1 resulting from the cross between red and blue morphs. C, D: offspring resulting from the backcross between the F1 and the blue morph acting as pollen receiver (C) or pollen donnor (D). E, F: offspring resulting from the backcross between the F1 and the red morph acting as pollen receiver (E) or pollen donnor (F). G: F2 offspring resulting from self-pollination of F1. Means and standard deviations are shown. Red: in this figure, this term includes plants with red flowers, salmon flowers and salmon-reddish flowers in human vision. Blue: Plants with blue flowers in human vision.

Flower colour characterization

The blue morph of *L. arvensis* reflected mainly in the ultraviolet, violet and blue, in the range between 330-450 nm. In contrast, the red morph reflected in the spectrum for yellow, orange and red (600-700 nm) with a reflectance peak in the ultraviolet (350 nm; Fig. 3). In the hexagon model, the blue morph was found within the UV-Blue sector and the red in the UV sector (Fig. 4). Both colour morphs were clearly separated from the center of the hexagon and from each other.

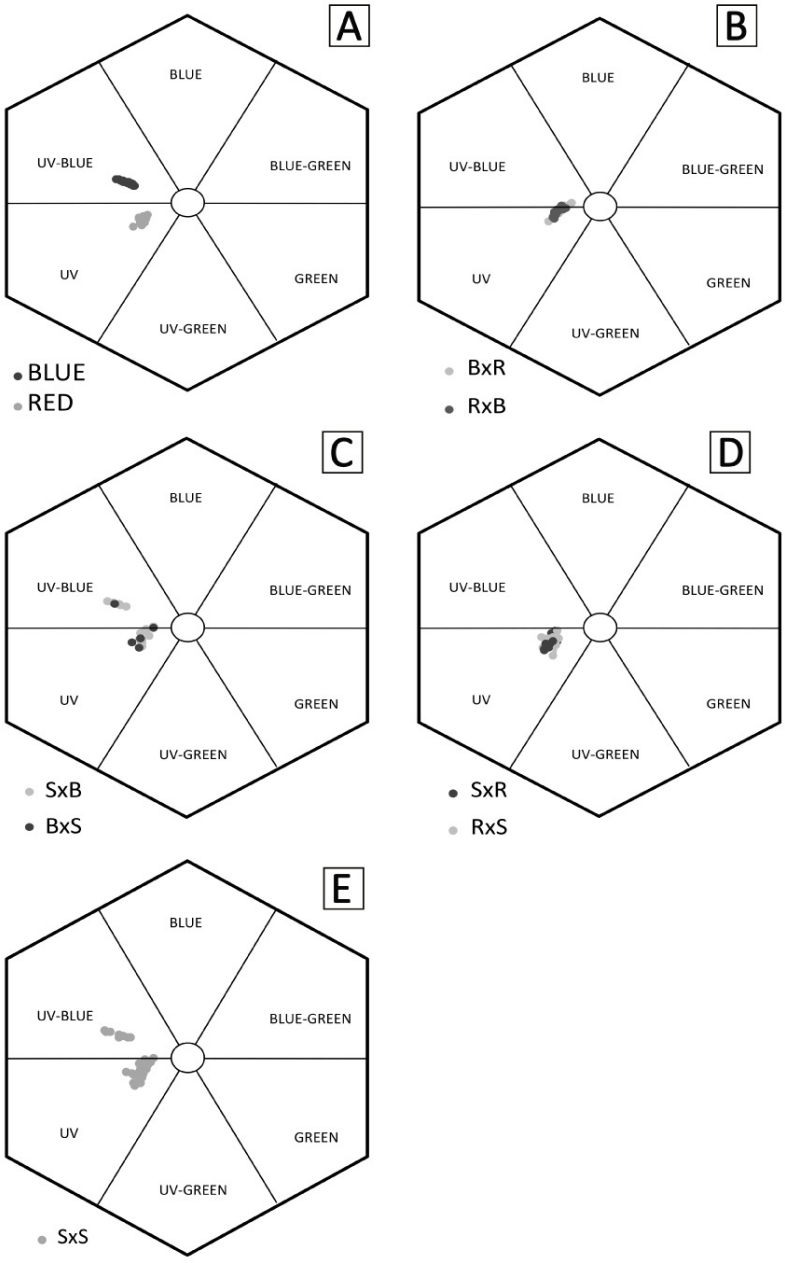

Figure 3. Representation of the flower colour of *Lysimachia arvensis* in the hexagon model proposed by Chittka (1992) based on the perception of color by bees. A: Blue and red morphs. B: F1 resulting from the cross between blue and red morphs. C: backcross between F1 and the blue morph. D: backcross between the F1 and the red morph. E: F2 offspring resulting from self-pollination of F1.

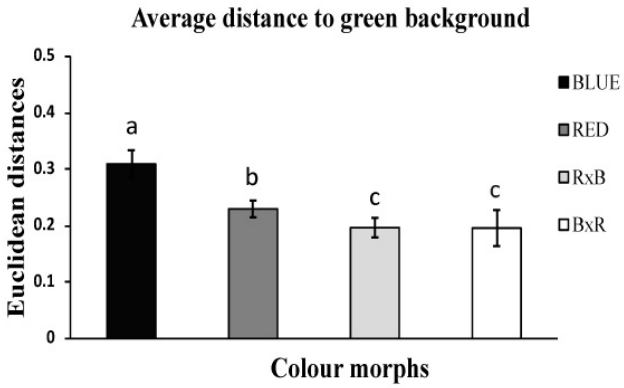

Figure 4. Mean Euclidean distances and standard deviation of the blue, red and F1 salmon morphs of L. arvensis to the center of the hexagon. The dashed line indicates the limit under which pollinators are not able to differentiate the flowers from the background. Different letters denote significant differences between the distances to the center of each morph (Tukey, $p < 0.05$).

The F1 showed peaks of reflectance very similar to those of the red parent, although with higher reflectance in the blue-violet wavelength (Fig. 3). The spectra of all F1 individuals were virtually identical regardless of the direction of the crossing (BxR or RxB). The F1 flowers were placed in the UV sector of the hexagon (Fig. 4), very close and even overlapped with the UV-Blue sector. This F1 was clearly separated from the center of the hexagon. The spectra of the F2 obtained by self-pollination of the F1 with (N = 41) appeared separated into two large groups, one similar to the blue morphotype (N = 8) and another similar to that of the red or salmon flowers (N = 33, Fig. 3). When the F2 was represented in the colour hexagon, eight plants coincided with the blue parent and the rest were placed in the area between red and the F1salmon (Fig. 4). Both groups were clearly differentiated from the center and from each other.

The backcross between the F1 salmon and the blue parent resulted in two groups of plants, one was in the UV-Blue sector of the hexagon and the other in the UV sector with a small part of the UV-Blue sector (Fig. 4). Individuals from both the BxS and SxB crossings, appeared in one of the two sectors UV or UV-Blue. These groups of plants were clearly separated from each other and with the center of the hexagon.

The reflectance spectra of the offspring from the backcross between the F1 salmon and the red morph, in either of the two senses, was the same as those of the red and salmon flowers (Fig. 4). When the red morph acted as a pollen receiver a more heterogeneus range of spectra appeared in the F2 than when the red morph acted as pollen donor. All the offspring from the crosses were found in the UV sector of the hexagon (Fig. 3D), although some of them were also located near the UV-Blue sector. In all cases the flowers were clearly separated from the center of the hexagon.

Significant differences were found in the Euclidean distances between blue, red, and salmon morphotypes (ANOVA, F3,122 = 204.801, p <0.001). The blue flowers showed the largest distances to the center of the hexagon and the salmon F1 the shortest. All distances were greater than 0.1 (Fig. 5).

The distance of the blue flowers to the red or to the salmon F1 was greater than 0.1 (Fig. 5). Likewise, the intramorph variability was very small in the blue morph, where the Euclidean distances were always lower than 0.1 among the measured flowers. The red morphotype showed a mean distance higher than 0.1 with the blue morph but lower than 0.1 with both other flowers of the same morph and with the salmon F1 (Fig. 5). Finally, salmon F1 flowers only showed distances higher than 0.1 with the blue morph (Fig. 5).

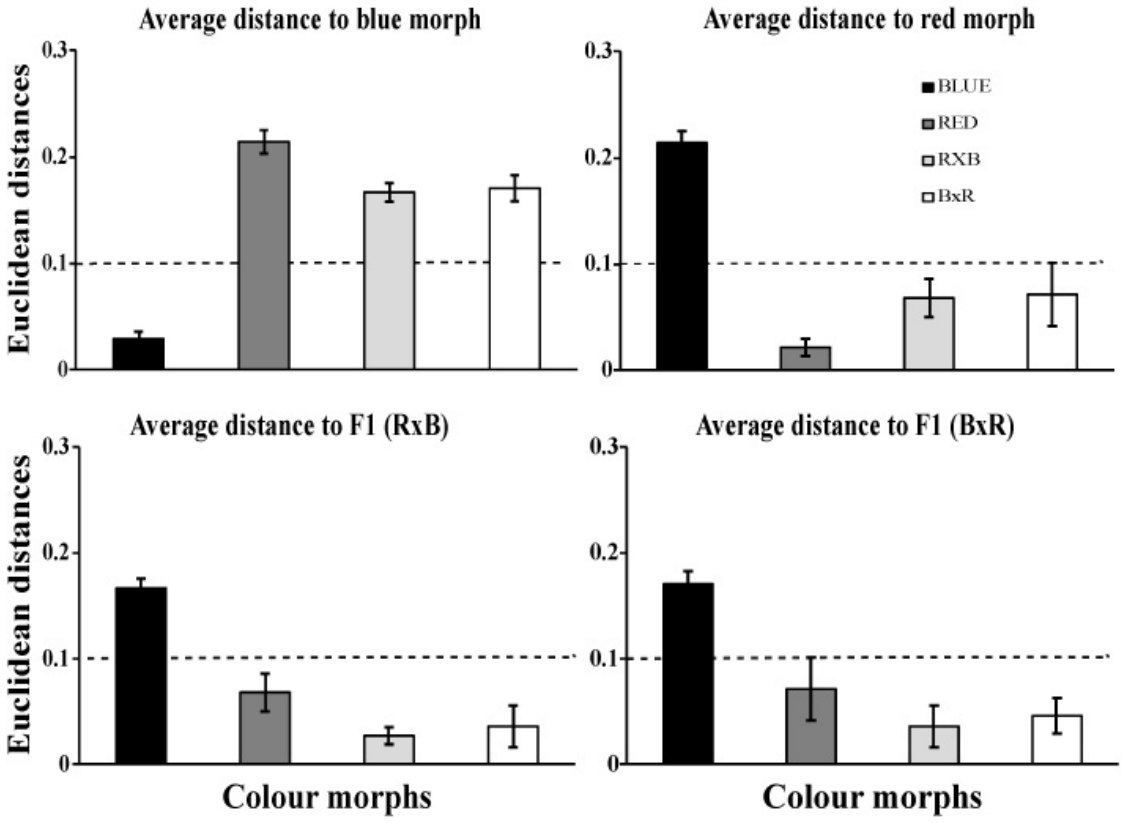

Figure 5. Mean Euclidean distances between the blue, the red and the F1 salmon morphs of *L. arvensis*. A: Distances to the blue morph. B: Distances to the red morph. C: Distances to the F1 salmon (red x blue) D: Distances to the F1 salmon (blue x red).

DISCUSSION

The results obtained in this work clearly indicate that the plants with salmon flowers results from the crossing between the pure red and blue morphotypes of *L. arvensis*. The F1 obtained was 100% homogeneous and showed an intermediate colouration between those of their two parents. This result indicates that there is no dominance-recessivity relationship

between the colour alleles of L. arvensis, as was previously described (Marsden-Jones & Weiss 1938), a codominance situation being more likely. It is possible that in some lighting circumstances, these salmon plants could be categorized as "red" in human vision, possibly leading to Weiss (1910) and to Marsden-Jones & Weiss (1938) to assume that the red allele was dominant. In fact, the quantitative measures of colour place these salmon plants very close to the red ones, although with a clearly different pattern.

The fact that F1 is homogeneous in colour suggests that the colour of the flowers in L. arvensis follows a characteristic segregation of a monogenic character, as described for floral colour in other species (Malerba and Nattero 2012). In fact, in the backcross between the blue morphotype and the F1, the proportion of individuals with blue flowers was 0.33. Likewise, in the F2 the proportion of individuals with blue flowers was 0.20, similar to that obtained in the cross between heterozygotes (0.25) of a monogenic character. However, the remaining individuals obtained in these crosses showed flowers which colours ranged from salmon to red, and they were clearly differentiated in human vision. This variation could indicate that there is more than one gene involved in the flower colour segregation in this species. However, L. arvensis is a tetraploid and the colour segregation obtained could be adjusted to the presence of two copies of the same gene (four alleles) in each individual. Thus, pure lines used as parental would have four alleles for the blue or red colour and the F1 would have two alleles of each colour giving rise to a phenotype with an intermediate colour between blue and red. The F2 obtained from self-pollination of the F1 would originate pure blue plants and pure red plants at a frequency of 1/16 each, salmon plants at a frequency of 6/16, and plants intermediate in colour at a frequency of 8/16. The proportions obtained experimentally do not match with these frequencies, being blue and red plants much more frequent than expected and intermediate plants much less frequent. In Lysimachia monelli, a sister species of L. arvensis with the same colour polymorphism, manual crosses between pure lines of blue and red flower plants give rise to an F1 similar to the red progenitor, but in F2 a third morphotype with pink flowers that differ subtly from red ones appears (Freyre and Griesbach 2004). The authors proposed a model of three genes to explain the inheritance of floral colour in this species. In our case, the results obtained are not adjusted to the three gene model, but neither to any other segregation based on simple models of few genes, with or without epistatic interactions between them. Thus, the number of genes responsibles for the flower colour in L. arvensis and the relationship between the genes remain unsolved.

Taking into account that anthocyanins are responsible for the colour of both the blue and the red flowers of *L. arvensis* (Harbone 1968), colour differences could be due to mutations of structural and/or regulatory genes of the biosynthetic pathway of these pigments. In flowers with anthocyanins, transitions from blue to red are relatively frequent (Rausher 2008) and are usually produced by the inactivation of some of the two genes F3'5'h or F3'h of the

anthocyanin pathway (Zufall and Rausher 2004; Rausher 2008). In the studied cases as those of Penstemon (Wessinger and Rausher 2013), Antirrhinum (Ishiguro et al. 2011), Phlox (Hopkins and Rausher 2011), Hibiscus (Gettys 2012) or Silene (Casimiro-Soriguer et al. 2016), are the F3'5'H coding genes and their regulators are responsible for the colour change. In these cases, a difference in the expression of a regulatory gene causes differences in the concentration of the anthocyanins which results in variations in the flower colour intensity. This kind of variation has appeared in the F2 offspring of *L. arvensis* and could indicate that some regulatory gene in the anthocyanin pathway could be involved in the expression of the floral colour, although this possibility would require a transcriptomic study of floral colour in *L. arvensis.*

Regarding the perception of the colours of the *L. arvensis* flowers by pollinators, as already verified by Ortiz et al. (2015), blue and red morphotypes are clearly distinguishable by bees since they perceive blue flowers as UV-blue and red flowers as UV. On the other hand, bees could also distinguish salmon individuals (F1) since their colour spectrum is mostly in the UV sector of the colour hexagon and clearly contrast with the green background. The average Euclidean distances between the red and the salmon flowers was less than 0.1, showing a continuum in the colour hexagon: This suggests that bees do not seem to be able to distinguish red and salmon flowers by petal colour. However, the salmon flowers have a greater bullseye than that of the red morph (Fig. 1), which could contribute to the its differentiation. The bulleseye of the flowers usually absorbs completely the ultraviolet radiation contrasting strongly with the rest of the petal (Van der Kooi et al. 2018); so that, its large size could improve the perception of salmon flowers over long distances (Koski and Ashman 2014). On the other hand, the blue morph showed the higher distance to the center of the hexagon (0.31 vs. 0.23 in the red and vs. 0.20 in the salmon) and so the greater contrast with the background, which is determinant for a quick detection of the flowers by pollinators (Chittka et al. 2001). Therefore, pollinators would be able to distinguish more easily the blue flowers than red or salmon flowers. In fact, in a previous work a greater preference of pollinators for blue flowers was already shown (Ortiz et al. 2015).

The fact that in the mixed natural populations individuals with salmon flowers hardly appear, indicates that the crossing between blue and red morphs rarely occurs. Our study show that "Flower colour" could be used in L. arvensis as a natural marker to determine both the rate of crossing between morphs and that of salmon individuals with their parents. The absence of plants with flowers of colour as found in in the F1 or the F2 in this study would indicate a reproductive isolation of blue and red morphs in natural populations of L. arvensis.

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REFERENCES

Arista M, Talavera M, Berjano R, Ortiz PL. 2013. Abiotic factors may explain the geographical distribution of flower colour morphs and the maintenance of colour polymorphism in the scarlet pimpernel. *J Ecol.* **101**: 1613-1622.

Casimiro-Soriguer I, Narbona E, Buide ML, del Valle JC, Whittall JB. 2016. Transcriptome and biochemical analysis of a flower color polymorphism in *Silene littorea* (*Caryophyllaceae*). *Front plant sci.* **7**: 204.

Chittka L. 1992. The colour hexagon: a chromaticity diagram based on photoreceptor excitations as a generalized representation of colour opponency. J Comp Physiol A: *Neuroethol Sens Neural & Behavior Physiolog.* **170**: 533-543.

Chittka L, Kevan PG. 2005. Flower colour as advertisement. En Dafni, A., Kevan, P. & Husband, B. C. (Eds.). Practical Pollination Biology. Enviroquest Ltd.: Canada. 157-196.

Chittka L, Menzel R. 1992. The evolutionary adaptation of flower colours and the insect pollinators' colour vision. *J Comp Physiol A: Neuroethol Sens Neural & Behavior Physiolog.* **171**: 171-181.

Chittka L, Spathe J, Schmidt A, Hickelsberger A. 2001. Adaptation, constraint and chance in the evolution of flower colour and pollinator colour vision. In Thomson JD. (Ed.). Cognitive Ecology of Pollination. Cambridge University Press: Cambridge (UK). 106–126.

Durbin ML, Lundy KE, Morrell PL, Torres-Martinez CL, Clegg MT. 2003. Genes that determine flower color: the role of regulatory changes in the evolution of phenotypic adaptations. *Mol phylogenetics evol.* **29**: 507-518.

Faegri K, van der Pijl L. 1966. The principles of pollination ecology. Pergamon Press. London (UK).

Fenster CB, Armbruster WS, Wilson P, Dudash MR, Thomson DJ. 2004. Pollination syndromes and floral specialization. *Ann Rev Ecol Evol Syst.* **35**: 375–403.

Freyre R, Griesbach RJ. 2004. Inheritance of flower color in *Anagallis monelli* L. *HortScience.* 39**:** 1220-1223.

Gettys LA. 2012. Genetic Control of White Flower Color in Scarlet Rosemallow (*Hibiscus coccineus* Walter). *J Hered.***103**: 594-597.

Grant V. 1993. Effects of hybridization and selection on floral isolation. *Proc Natl Acad Sci.* **90**: 990-993.

Harborne JB. 1968. Comparative biochemistry of the flavonoids—VII: Correlations between flavonoid pigmentation and systematics in the family *Primulaceae*. *Phytochem.* **7**: 1215-1230.

Hoffmann HKH. 1879. Nachträge zur Flora des Mittelrhein-Gebietes. Schmitz.

Hopkins R, Rausher MD. 2011. Identification of two genes causing reinforcement in the Texas wildflower Phlox drummondii. *Nat.* 469, 411.

Huxley JS. 1955. Morphism and evolution. *Hered.* **9**: 1-51.

Ishiguro K, Taniguchi M, Tanaka Y. 2012. Functional analysis of Antirrhinum kelloggii flavonoid 3′-hydroxylase and flavonoid 3′, 5′-hydroxylase genes; critical role in flower color and evolution in the genus *Antirrhinum*. *J plant res.* **125**: 451-456.

Ishikura N. 1981. Flavonoids in the petal cells of *Anagallis arvensis* f. *coerulea* containing a blue crystalline anthocyanin. *Zeitschrift für Pflanzenphysiologie* **103**: 469-473.

Kay QON, Daoud HS, Stirton CH. 1981. Pigment distribution, light reflection and cell structure in petals. *Bot J Linn Soc.* **83**: 57–83.

Koski MH, Ashman TL. 2014. Dissecting pollinator responses to a ubiquitous ultraviolet floral pattern in the wild. *Funct Ecol.* **28**: 868–877.

Lagomarsino LP, Forrestel EJ, Muchhala N, Davis CC. 2017. Repeated evolution of vertebrate pollination syndromes in a recently diverged Andean plant clade. *Evol.* **71(8)**: 1970-1985.

Lin-Wang K, Bolitho K, Grafton K, Kortstee A, Karunairetnam S, McGhie TK, Allan AC. 2010. An R2R3 MYB transcription factor associated with regulation of the anthocyanin biosynthetic pathway in Rosaceae. *BMC plant biol.* **10**: 50.

Malerba R, Nattero J. 2012. Pollinator response to flower color polymorphism and floral display in a plant with a single-locus floral color polymorphism: consequences for plant reproduction. *Ecol Res.* **27**: 377-385.

Marsden-Jones EM, Weiss FE. 1938. The essential differences between *Anagallis arvensis* L. and *Anagallis foemina* Mill. *Proc Linn Soc Lond.* **150**: 146-155.

Narbona E, Wang H, Ortiz PL, Arista M, Imbert E. 2018. Flower colour polymorphism in the Mediterranean Basin: occurrence, maintenance and implications for speciation. *Plant Biol.* **20**: 8-20.

Ortiz PL, Berjano R, Talavera M, Rodríguez-Zayas L, Arista M. 2015. Flower colour polymorphism in Lysimachia arvensis: How is the red morph maintained in Mediterranean environments? *Perspect Plant Ecol Evol Syst.* **17**: 142-150.

Pax F, 1905. *Primulaceae*/*Primula*. In Engler A. (ed.). Das Pflanzenreich, Band IV/237, H. R. Engelmann, Leipzig, Germany. 1–160.

Rausher MD, Miller RE, Tiffin P. 1999. Patterns of evolutionary rate variation among genes of the anthocyanin biosynthetic pathway. *Mol biol evol.* **16(2)**: 266-274.

Rausher MD. 2008. Evolutionary transitions in floral color. *Int J Plant Sci.* **169**: 7-21.

Servedio MR, Van Doorn GS, Kopp M, Frame AM, Nosil P. 2011. Magic traits in speciation: 'magic' but not rare? *Trends Ecol Evol.* **26**: 389-397.

Smith SD, Rausher MD. 2011. Gene loss and parallel evolution contribute to species difference in flower color. Mol Biol Evol. **28(10)**: 2799-2810.

van der Kooi CJ, Elzenga JTM, Staal M, Stavenga DG. 2016. How to color a flower: on the optical principles of flower coloration. *Proc R Soc B: Biol Sci.* **283**: 20160429.

van der Kooi CJ, Dyer AG, Kevan PG, Lunau K. 2018. Functional significance of the optical properties of flowers for visual signalling. *Ann bot. doi:* **10**.1093/aob/mcy119

Weiss MR. 1995. Floral color change: a widespread functional convergence. *Am J Bot.* 167-185.

Wessinger CA, Rausher MD. 2012. Lessons from flower colour evolution on targets of selection. *J Exp Bot.* **63**: 5741-5749.

Wessinger CA, Rausher MD. 2014. Predictability and irreversibility of genetic changes associated with flower color evolution in Penstemon barbatus. *Evol.* **68**: 1058-1070.

Zhang Y, Butelli E, Martin C. 2014. Engineering anthocyanin biosynthesis in plants. *Curr opin plant biol.* **19**: 81-90.

Zimmermann IM, Heim MA, Weisshaar B, Uhrig JF. 2004. Comprehensive identification of *Arabidopsis thaliana* MYB transcription factors interacting with R/B-like BHLH proteins. *T Plant J.* **40**: 22-34.

Zufall RA, Rausher MD. 2004. Genetic changes associated with floral adaptation restrict future evolutionary potential. *Nat.* **428**: 847–850.

3. Nuclear microsatellite primers in the annual herb *Lysimachia arvensis* (Myrsinaceae) and closely related taxa.

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Rousset, F., **2008.** Genepop'007: A complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources*, 8, 103–106.

Sánchez-Robles, J. M., Balao, F., García-Castaño, J. L., Terrab, A., Navarro-Sampedro, L. & Talavera, S., **2012.** Nuclear microsatellite primers for the endangered relict fir, Abies pinsapo (pinaceae) and cross-amplification in related mediterranean species. *International journal of molecular sciences*, 13, 14243-14250.

Schuelke, M., **2000.** An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology*, 18, 233–234.

Van Puyvelde, K., Van Geert, A. & Triest, L., **2010.** ATETRA, a new software program to analyze tetraploid microsatellite data: comparison with TETRA and TETRASAT. *Molecular Ecology Resources*, 10, 331-334.

Nuclear Microsatellite Primers in the Annual Herb *Lysimachia arvensis* (Myrsinaceae) and Closely Related Taxa

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Abstract: The maintenance of flower color polymorphism in Mediterranean populations of *Lysimachia arvensis* is enigmatic due to the existence of both biotic and abiotic pressures against the red morph. The mixed reproductive system of *L. arvensis* could offer reproductive assurance protecting the red morph. To test this hypothesis we have developed thirteen nuclear microsatellite primers that were applied to ten individuals from two red-flowered populations of *L. arvensis*. Ten loci were polymorphic and the number of alleles ranged from two to ten. High levels of inbreeding (estimated by F_{IS}) in red-flowered *L. arvensis* were shown. Cross-amplification was tested in other closely related taxa and was successful in most cases. These microsatellite primers provide tools to investigate the importance of delayed selfing, genetic diversity and population structure in *L. arvensis* and closely related taxa.

Keywords: *Lysimachia*, nSSR, Floral Color Polymorphism, Tetraploid, *Anagallis*, Inbreeding.

1. Introduction

Lysimachia arvensis (L.) U. Manns & Anderb. is a widely distributed annual herb with floral color polymorphism (blue and red) which shows a geographical pattern associated with environmental conditions. The blue morph shows higher fitness in Mediterranean environments, while the red morph performs better in wet places (Arista et al., 2013). Moreover, in Mediterranean populations, pollinators exert a strong directional phenotypic selection for blue flowered plants (Ortiz et al., in press). Despite both abiotic and biotic selective pressures against the red morph, it frequently appears in Mediterranean populations, although in low proportion. The maintenance of flower color polymorphism in Mediterranean populations is enigmatic because selection against the red morph combined with random genetic drift usually leads to their rapid loss. In *Lysimachia arvensis* flowers can receive cross pollen during the two first days of anthesis, but at the end of the anthesis they show delayed selfing (Gibbs & Talavera, 2001; Ortiz et al., in press). This mixed reproductive system could offer reproductive assurance when outcrossed pollen is limited, mitigating the effects of both biotic and abiotic selective agents and protecting the red morph. However, this needs to be proved by using molecular data. We have developed nuclear microsatellite markers for *L. arvensis* to investigate its mating system, genetic diversity and population structure in Mediterranean environments. Furthermore, the developed microsatellites were tested in closely related taxa.

2. Material and Methods

2.1 Plant Material

Lysimachia arvensis (former *Anagallis arvensis* L.) is an annual, tetraploid herb probably from the Mediterranean Basin, although now it is distributed worldwide. In taxonomic studies, the presence of flower color polymorphism gave rise to the description of two subspecies: the typical red-flowered plants and the subsp. *caerulea* (L.) Cout. in plants with blue flowers. Microsatellites were developed for the red flowered plants of *L. arvensis,* and were tested in the blue-flowered plants and four other closely related taxa. These taxa were 1) *Lysimachia foemina* (Mill.) U. Manns & Anderb., an annual tetraploid species with blue flowers, previously included in *L. arvensis* as a subspecies, but now considered a different species (Manns & Anderberg, 2007), 2) *Anagallis parviflora* Hoffmanns. & Link an annual diploid taxon with very small flowers, sometimes included as a subspecies of *Anagallis arvensis* (now *L. arvensis*), 3) *Lysimachia monelli* (L.) U. Manns & Anderb (former *Anagallis monelli* L.,) is a perennial diploid species with two color morphs, described as subspecies: the typical with blue flowers and subsp.

collina (Schousb.) Maire with orange flowers (Pujadas, 1989); and 4) *Lysimachia nemorum* L., a perennial diploid species with yellow flowers that was always placed in the genus *Lysimachia*.

We collected two populations of *L. arvensis* with red-flowered plants, one from Carcabuey (arv-A, Cordoba, Spain, SEV279258) and another native to Cies Islands (arv-B, Pontevedra, Spain, SEV248969). The nSSRs were tested in 10 individuals from each population. In addition, cross-amplification was tested in 5 individuals of one population for each of the following taxa: *L. arvensis* with blue flowers (cae) collected in Zahara de los Atunes (Cádiz, Spain, SEV278757), *L. monelli* with blue flowers (mon) collected in Aracena (Huelva, Spain, SEV279279), *L. monelli* var. *collina* (col) collected in Mont-Roig (Tarragona, Spain, SEV285113), *L. foemina* (foe) collected in Sierra Mágina (Jaen, Spain, SEV279153), *A. parviflora* (par) collected in Odemira (Alentejo, Portugal, SEV284451) and *L. nemorum* (nem) collected in Terceira (Azores Islands, Portugal, SEV275589). Vouchers for all the studied taxa were deposited at the Herbarium of the Seville University.

2.2 DNA Extraction, and Microsatellite Discovery

Total genomic DNA was isolated from dry leaf tissue with a plant extraction kit (Invisorb Spin Plant Mini Kit, Invitek, Berlin, Germany) following the supplier's instructions. The DNA samples were enriched for microsatellites by building a DNA library using Dynabeads, as described in Glenn and Schable (2005), and sequenced on a 454 Genome Sequencer FLX System (454 Life Sciences, a Roche company, Branford, CT, USA) at the Savannah River Ecology Laboratory (Aiken, SC, USA). The screening sequence data and the design of primers were made following the procedures described in Sánchez-Robles et al. (2012). CAP3 was used to assemble sequences at 98% sequence identity using a minimal overlap of 75 bp. Sequence data were screened for di-, tri- and tetra-nucleotide repeats using the program MSATCOMMANDER version 0.8.1 with minimum repeats set to eight, seven and six respectively, and primers were designed with Primer3. To allow a cheaper labeling technique than the direct labeling of the primers (Schuelke, 2000), two default 5'-tails (CAG: 5'-CAGTCGGGCGTCATCA-3' and M13R: 5'-GGAAA CAGCTATGACCAT-3') options were considered for designed primers, as with Faircloth (2008). The inclusion of the 5'-tail make possible the use of a third primer fluorescently labeled in the polymerase chain reaction (M13R or CAG), for detection in the DNA Analyzer sequencer (Boutin-Ganache et al., 2001). Futhermore, a not-tagged primer from a primer pair was designed with a 5'-GTTT tail to provide adenylation and facilitate genotyping (Brownstein et al., 1996). Primers could be designed for 305 of the 1551 sequences obtained. Of these 305 primer pairs, we discarded 196 with low quality. Therefore, a total of 109 high quality primers pairs were obtained.

2.3 PCR Amplification and Quality Test

Primers with tri- and tetra-nucleotide repeats were selected. To test the amplification quality, preliminary screenings of 47 loci were done for eight samples in collaboration with GenoScreen. From these, 19 primer pairs produced clear high-quality patterns in the agarose gel. PCR amplification was carried out under the following conditions, modified from the Sanchez-Robles protocol (2012): an initial denaturation step of 10 min at 95 °C, followed by five cycles of 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s; next step of 11 cycles of 94 °C for 30 s, 65 °C for 30 s (decreased by 0.5 °C per cycle), and 72 °C for 1 min; then 30 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min; and a final step of 10 min at 72 °C. The PCR reactions were carried out using approximately 20 ng of genomic DNA in a total volume of 20 µL, containing 2 µL of PCR Buffer 1× 0,4µL of MgCl2 3mM, 0,2 µL of dNTP 1 mM (0.25 mM each), 0.5 µL of BSA (0.05 mg/mL), 0.2 µL of i-Start Taq DNA polymerase (1 U/µL) (iNtRON Biotecnology Inc., Sungnam, Korea), 1.25 µL of primer with 5'-GTTT tail (0.25 µM), 0.3 µL of primer with 5'-CAG or M13R tail (0.06 µM) and 0.5 µL of universal primer with 6-carboxyfluorescein (FAM), nitrobenzoxadiazolyl (NED), or VIC fluorescent label (0.25 µM). The PCR products were run on a 3730 DNA Analyzer sequencer (Applied Biosystem, Foster City, CA, USA) and sized with LIZ 500 standard (Applied Biosystem, Foster City, CA, USA) and thirteen markers with good quality profiles were selected (Table 1). Fragments were analyzed using GENEMARKER version 1.9 (SoftGenetics, State College, PA, USA).

We estimated expected heterozygosity (H_E) with ATETRA version 1.2 (Van Puyvelde, Van Geert & Triest, 2010), which takes into account all possible combinations of allele copy numbers in populations with partial heterozygotes (Van Puyvelde et al. 2010). Both the observed heterozygosity (H_O), Hardy-Weinberg equilibrium test (HWE) and the inbreeding coefficient (F_{IS}) were estimated by using GENODIVE, version 2.0b25 (Meirmans & Van Tienderen, 2004). Null allele frequencies (*Na*) were calculated by using the R Package POLYSAT version 1.10 (Clark & Jasieniuk, 2011) for tetraploid taxa, and by using GENEPOP 4.0.10 software for diploid taxa. Owing to the lack of a software to compute linkage disequilibrium (LD) for tetraploid data, ploidy level was reduced to diploid data by using GENODIVE, then LD was tested for all possible combinations of loci by using Fisher's exact test in GENEPOP (Raymond & Rousset, 1995; Rousset, 2008).

3. Results and Discussion

Three of the thirteen high-quality loci tested in 20 individuals from two populations of red-flowered plants of *L. arvensis* were monomorphic (*Lys11*, *Lys16* and *Lys23*; Table 1). The remaining ten loci were polymorphic, and the number of alleles ranged from two to ten (mean ± SE: 4.400 ± 0.763). When analyzing the two populations separately, the loci Lys 12, Lys 17, Lys 24 and Lys 28 were also monomorphic in arv-B (Table 2). In the polymorphic loci, observed/expected heterozygosities ranged from 0.233-0.841/0.180-0.822 in arv-A and from 0.053-0.676/0.094-0.652 in arv-B, and inbreeding coefficient ranged from -0.037 to 0.903 in arv-A and from -0.274 to 0.764 in arv-B (Table 2). Null allele frequencies (*N*a) ranged from 0.010 to 0.645 in arv-A and from 0.024 to 0.573 en arv-B (Table 2). In the two populations studied, most loci exhibited deviation from Hardy-Weinberg proportions, while only one pairs of loci (Lys17/Lys45) exhibited significant linkage disequilibrium ($P \leq 0.05$). Lys 29 was the only locus with negative values in both populations, showing unbalanced outbreeding. Lys 28 in arv-A and Lys 31 in arv-B were nearly balanced between selfing and outcrossing.

Table 1.- Details of 13 novel nuclear microsatellite primers developed in *Lysimachia arvensis*

Locus	Primer sequences (5'-3')	Repeat motif	Size range (bp)	Ta (°C)	A	GenBank Accession Nº
Lys11	F:***CTTCATTCTTGGTGGTTCTG R:**CATTCAGGAACCTACACACC	ACGC(10)	175	60	1	KP081754
Lys12	F:*CTGACCTCCTGTTCCAATC R:***TGTGGGCAATAATATTCTCC	ATC(9)	238-252	60	3	KP081755
Lys16	F:***ACTGCGATGATTTATGAACC R:*GCTTCCAATTGATGTCTCTG	ACAT(14)	187	60	1	KP081756
Lys17	F:***CACCCATAGCATCTTACTTG R:*TTGCCACAATGTGTAACTG	AGAT(6)	300-308	60	2	KP081757
Lys23	F:**ATTTCATCTTCACATCCAATG R:***AAACAAGCTCTGCAAAGAAC	ACAT(6)	207	60	1	KP081758
Lys24	F:*CCCATTACACATTGGTTTAAC R:***CTGACTGACGAGCATCTAATG	AAAG(7)	213-257	60	2	KP081759
Lys28	F:***AAACCATGCTACTACGATCC R:*AACTCCTATCAAAGCCAAAG	AAAG(6)	160-180	60	4	KP081760
Lys29	F:***CACCTCGTTGCTATTGACTC R:*AAAGTGAAGGGACGATGTC	AAAG(9)	172-201	60	4	KP081761
Lys30	F:**AATCACTCCCACCAGATTC R:***TGGGTGGTTTATCAATTGAC	AGAT(10)	186-215	60	6	KP081762
Lys31	F:**TCAGTCAGTCAGTCCACAGC R:***AATCTTGGATCGGATAAGTG	AAAG(7)	190-202	60	4	KP081763
Lys32	F:*CCTCCTCATCACAAATTCAC R:***GCTCGTTGACTTGTGACTTC	AAAG(14)	216-246	60	10	KP081764
Lys33	F:**CAGTCAGTTCCATGATCACC R:***AGATAGCCTGCAAAGAGATG	AATG(6)	276-289	60	6	KP081765
Lys45	F:***CTAAGGAGGAAGCACATTTG R:**CAAGAAACCTAAACGAAATTG	ATC(9)	278-300	60	3	KP081766

For each marker, the forward (F) and the reverse (R) sequence are given. T_a = annealing temperature; *A* = number of alleles; * indicates M13R tag (5'-GGAAACAGCTATGACCAT-3'), ** indicates CAG tag (5'-CAGTCGGGCGTCATCA-3'), *** indicates GTTT tag.

Table 2: Results of primer screening in two *Lysimachia arvensis* populations with red-flowered plants.

	Arv-A (N=10)						Arv-B (N=10)					
Locus	A	Ho	He	F_{IS}	p-value	Na	A	Ho	He	F_{IS}	p-value	Na
Lys11	1	0.000	0.000	-	-	-	1	0.000	0.000	-	-	-
Lys12	3	0.279	0.338	0.774	0.001*	0.463	1	0.000	0.000	-	-	-
Lys16	1	0.000	0.000	-	-	-	1	0.000	0.000	-	-	-
Lys17	2	0.370	0.320	0.755	0.001*	0.583	1	0.000	0.000	-	-	-
Lys23	1	0.000	0.000	-	-	-	1	0.000	0.000	-	-	-
Lys24	2	0.233	0.180	0.791	0.001*	0.506	1	0.000	0.000	-	-	-
Lys28	4	0.588	0.595	-0.037	0.393	0.070	1	0.000	0.000	-	-	-
Lys29	4	0.647	0.631	-0.216	0.005	0.010	2	0.513	0.492	-0.274	0.016	0.024
Lys30	6	0.777	0.771	0.377	0.001*	0.347	3	0.349	0.338	0.725	0.001*	0.498
Lys31	2	0.461	0.480	0.903	0.001*	0.645	3	0.249	0.314	0.071	0.303	0.106
Lys32	6	0.841	0.822	0.390	0.001*	0.358	4	0.676	0.652	0.163	0.052	0.242
Lys33	4	0.547	0.578	0.854	0.001*	0.587	2	0.053	0.094	0.764	0.001*	0.202
Lys45	2	0.369	0.320	0.870	0.001*	0.583	3	0.647	0.632	0.319	0.001*	0.573

- *N* = simple size; *A* = number of alleles; *Ho* = observed heterozygosity; *He* = expected heterozygosity; F_{IS}=inbreeding coefficient. Positive values of *F*IS (maximum = 1) indicate an excess of homozygotes and negative values (maximum = −1) indicate an excess of heterozygotes; * Significant deviation from Hardy–Weinberg equilibrium ($P \leq 0.001$); *Na* = null allele frequency.

Cross-amplification in the other studied taxa was successful in most cases (Table 3). Five primers (Lys12, Lys28, Lys29, Lys30 and Lys33) were amplified in all the taxa and, four other primers (Lys17, Lys31, Lys32 and Lys46) were amplified in the taxa formerly assigned to genus *Anagallis*. Most of the primers studied were polymorphic. The null allele frequencies observed for different loci indicate that it is likely an overestimation of genetic distances between the related taxa (Chapuis & Estoup, 2006) and, consequently, our results should be cautiously considered.

Table 3: Cross-amplification of 13 primers, developed in red-flowered plants of *Lysimachia arvensis,* in blue-flowered plants of this taxon and in four

		Taxa					
Locus		cae	foe	col	mon	par	nem
Lys11	I	0/5	0/5	1/5	1/5	0/5	0/5
	A	-	-	1	1	-	-
	Na	-	-	-	-	-	-
Lys12	I	5/5	5/5	5/5	5/5	5/5	3/5
	A	2	3	1	4	1	2
	Na	0.505	0.464	-	0.268	-	0.577
Lys16	I	0/5	0/5	1/5	1/5	2/5	0/5
	A	-	-	1	1	1	-
	Na	-	-	-	-	-	-
Lys17	I	5/5	2/5	2/5	2/5	5/5	0/5
	A	1	1	2	2	3	-
	Na	-	-	0.707	0.707	0.508	-
Lys23	I	0/5	0/5	0/5	0/5	1/5	0/5
	A	-	-	-	-	1	-
	Na	-	-	-	-	-	-
Lys24	I	0/5	0/5	5/5	2/5	0/5	5/5
	A	-	-	3	2	-	2
	Na	-	-	0.663	0.707	-	0.000
Lys28	I	5/5	5/5	5/5	5/5	4/5	2/5
	A	3	4	4	4	3	2
	Na	0.409	0.224	0.236	0.000	0.728	0.000
Lys29	I	5/5	5/5	5/5	5/5	5/5	2/5
	A	4	4	3	3	4	2

	Na	0.245	0.412	0.055	0.327	0.000	0.000
Lys30	I	5/5	2/5	5/5	5/5	5/5	5/5
	A	5	2	3	2	5	6
	Na	0.136	0.556	0.787	0.632	0.459	0.000
Lys31	I	5/5	5/5	5/5	5/5	2/5	0/5
	A	3	4	4	2	1	-
	Na	0.463	0.224	0.429	0.000	-	-
Lys32	I	5/5	1/5	5/5	5/5	5/5	0/5
	A	3	1	4	2	3	-
	Na	0.385	-	0.197	0.000	0.377	-
Lys33	I	5/5	5/5	1/5	5/5	5/5	2/5
	A	3	5	1	2	1	1
	Na	0.494	0.053	-	0.632	-	-
Lys46	I	5/5	1/5	5/5	2/5	5/5	0/5
	A	2	1	2	1	2	-
	Na	0.568	-	0.894	-	0.000	-

Note: cae: blue-flowered plants of *L. arvensis*; foe: *L. foemina*, mon: blue-flowered plants of *L.monelli,* col: red-flowered plants of *L. monelli,* par: *Anagallis parviflora*; nem: *L. nemorum*. I= number of individuals that amplified in the locus, A= number of alleles, Na= null allele frequency

Tetraploid taxa usually present higher allele numbers and levels of heterozygosity than related diploid taxa (Hardy & Vekemans, 2001; Marrs, Sforza & Hufbauer, 2008; Ferriol et al., 2014). However, our results show that the tetraploid *L. arvensis* and *L. foemina* have a similar allele number to the diploid taxa (*L. monelli*, *A. parviflora* and *L. nemorum*). This occurs even when taking into account the two red-flowered populations of *L. arvensis,* where a higher number of individuals were studied. Our results also show high levels of inbreeding (estimated by FIS) in red-flowered *L. arvensis*, mainly in arv-B from Cies Islands. Therefore, in spite of the mixed mating system of *L. arvensis*, selfing seems more relevant than outcrossing in the populations studied. The nSSR developed could be used in future studies to clarify the role of the breeding system in genetic variation for *L. arvensis*.

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References

Abdelkrim, J., Roberston, B.C., Stanton, J.L. & Gemmell, N.J., **2009.** Fast, cost-effective development of species-specific microsatellite markers by genomic sequencing. *BioTechniques*, 46, 185–192.

Arista, M., Talavera, M., Berjano, R. & Ortiz, P. L., **2013.** Abiotic factors may explain the geographical distribution of flower colour morphs and the maintenance of colour polymorphism in the scarlet pimpernel. *Journal of Ecology*, 101, 1613-1622.

Boutin-Ganache, I., Raposo, M., Raymond, M. & Deschepper, C.F., **2001.** M13-tailed primers improve the readability and usability of microsatellite analyses performed with two different allele-sizing methods. *Biotechniques*, 31, 24–28.

Brownstein, M.J., Carpten J.D. & Smith, J.R., **1996.** Modulation of non-templated nucleotide addition by Taq DNA polymerase: Primer modifications that facilitate genotyping. *Biotechniques*, 20, 1004–1010.

Pujadas A. **1989**. *Anagallis* L. in Castroviejo, S., Aedo, C., Cirujano, S., Laínz, M., Montserrat, P., Morales, R., Muñoz Garmendia, F., Navarro, C., Paiva, J. & Soriano, C. (eds.): 57-62. Flora iberica 5. Real Jardín Botánico, CSIC, Madrid.

Chapuis, M.P. & Estoup A., **2007**. Microsatellite null alleles and estimation of population differentiation. *Molecular biology and evolution*, 24, 621-631.

Clark, L.V. & Jasieniuk, M., **2011. P**olysat: an R package for polyploid microsatellite analysis. *Molecular Ecology Resources*, 11, 562-566.

Ferriol, M., Merle, H. & Garmendia, A., **2014.** Microsatellite evidence for low genetic diversity and reproductive isolation in tetraploid Centaurea seridis (Asteraceae) coexisting with diploid *Centaurea aspera* and triploid hybrids in contact zones. *Botanical Journal of the Linnean Society*, *176*, 82-98.

Gibbs, P.E. & Talavera, S., **2001.** Breeding system studies with three species of *Anagallis* (Primulaceae): self-incompatibility and reduced female fertility in *A. monelli* L. *Annals of Botany*, 88, 139-144.

Glenn, T.C. & Schable, N.A., **2005.** Isolating microsatellite DNA loci. *Methods in Enzymology*, *395*, 202–222.

Hardy O.J. & Vekemans X., **2001.** Patterns of allozyme variation in diploid and tetraploid *Centaurea jacea* at different spatial scales. *Evolution*, 55, 943–954.

Lance, S.L., Light, J.E., Jones, K.L., Hagen, C. & Hafner, J.C., **2010.** Isolation and characterization of 17 polymorphic microsatellite loci in the kangaroo mouse, genus *Microdipodops* (Rodentia: Heteromyidae). *Conservation Genetics Resources*, 2, 139–141.

Gardner, M.G., Fitch, A.J., Bertozzi, T. & Lowe, A.J., **2011.** Rise of the machines—Recommendations for ecologists when using next generation sequencing for microsatellite development. *Molecular Ecology Resources*, 11, 1093–1101.

Faircloth, B.C., **2008.** Msatcommander: Detection of microsatellite repeat arrays and automated, locus-specific primer design. *Molecular Ecology Resources*, 8, 92–94.

Ortiz, P.L.; Berjano, R.; Talavera, M.; Rodríguez-Zayas L. & M. Arista.; In press. Flower color polymorphism in *Lysismachia arvensis*: how is the red morph maintained in Mediterranean environments? *Perspectives in Plant Ecology, Evolution and Systematics*.

Manns, U. & Anderberg, A. A., **2007.** Relationships of *Anagallis foemina* and *A. arvensis* (**Myrsinaceae**): New insights inferred from DNA sequence data. *Molecular Phylogenetics and Evolution*, *45*, 971-980.

Marrs R.A., Sforza R. & Hufbauer R.A., **2008.** When invasion increases population genetic structure: a study with *Centaurea diffusa*. *Biological Invasions***,** 10, 561–572.

Meirmans, P. G. & Van Tienderen, P. H., **2004.** GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms. *Molecular Ecology Notes*, *4*, 792-794.

Raymond, M. & Rousset, F., **1995.** GENEPOP: population genetics software for exact tests and ecumenicism. *Journal of heredity*, 86(3), 248-249.

4. Selfing maintains flower colour polymorphism in *L. arvensis* despite high inbreeding depression.

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(Jiménez-López F.J., Ortiz P. L., Talavera M. & Arista M. Flower colour polymorphism in *L. arvensis* despite high inbreeding depression. En preparación para mandar a …..)

ABSTRACT

Flower colour polymorphism (FCP) is frequently associated with differences in pollinator attraction. FCP maintenance is intriguing, as positive directional selection by pollinators should result in the loss of polymorphism. Autonomous selfing could confer reproductive assurance when pollen is limited, and could be a mechanism for maintaining polymorphism unless inbreeding depression is high. We study the role of selfing in maintaining FCP in *Lysimachia arvensis,* a species with blue and red morphs co-occurring in Mediterranean populations, where pollinators negatively select for the red morph. We experimentally assessed inbreeding depression in both morphs in two Mediterranean populations and genetic diversity was studied via AFLP and SSR microsatellites in 20 populations. Between-morph genetic differentiation was high and the red morph had a lower genetic diversity. Results also show strong phenological differences between selfed and outcrossed progeny, and a high ID of the red morph. The low genetic diversity of the red morph is in concordance with a reproductive system based predominantly on selfing. However, ID suggests a limited capacity for red morph recruitment, according to its low frequency in polymorphic populations. Genetic differentiation between morphs indicates a low gene flow between them, opening the possibility of reproductive isolation and speciation in *Lysimachia arvensis*.

KEY WORDS: *Anagallis*, gene flow, speciation, flower colour polymorphism, inbreeding depression, phenology

INTRODUCTION

Flower colour polymorphism (FCP) is defined as “the presence of at least two genetically-determined colour morphs within a single interbreeding population, the rarest of which is too frequent to be solely the result of recurrent mutation” (Huxley 1955). Flower colour polymorphism is a widespread trait in plants, but is relatively infrequent (e.g. Whitney 2005; Rausher 2008; Narbona et al. 2018). In many cases, both biotic and abiotic factors influence the fitness of plants with flowers of a certain colour. Thus, floral morphs can show differential tolerance to abiotic factors (Warren and Mackenzie 2001; Arista et al. 2013) due to the association between some floral pigments and protective flavonoids. Similarly, biotic agents of selection such as herbivores (Strauss and Whittall 2006; Sobral et al. 2015) or pollinators (e.g. Meléndez-Ackerman et al. 1997; Jones and Reithel 2001) can show a preference for a particular morph, thereby being responsible for its lower or higher fitness, respectively. Under these circumstances, the maintenance of flower colour polymorphism is an apparent enigma, as directional selection along with genetic drift should drive the loss of one of the colour morphs.

Pollinators are among the most important biotic factors involved in flower colour selection (e.g. Fenster et al. 2004; Whibley et al. 2006; Wessinger and Rausher 2012; Schiestl and Johnson 2013). They usually discriminate between colour morphs and can show preferences for a particular colour causing positive (e.g. Morgan and Schoen 1997; Jones and Reithel 2001; Ortiz et al. 2015), or negative directional selection (e.g. Waser and Price 1981, 1983; Gigord et al. 2001; Kagawa and Takimoto 2016). In polymorphic species, the less-visited morph could suffer a fitness reduction leading to negative directional selection, which along with genetic drift should result in the loss of polymorphism and the evolution of monomorphic populations (Waser and Price 1981; Levin and Brack 1995; Campbell et al. 1997; Jones and Reithel 2001). However, the less-visited morph will suffer fitness reduction only if it depends strictly on pollinators to reproduce. Some plants have the capacity to produce seeds by autonomous selfing when pollinators are scarce, thereby showing a mixed mating system. This capacity, called reproductive assurance (RA), allows plant reproduction when opportunities for outcrossing are reduced (Holsinger 2000), as occurs when pollinator attention is low.

Plants with reproductive assurance capacity via selfing can be independent of pollinators and can be maintained in populations, at least for some time (Takebayasi and Morrell 2001; Charlesworth 2006). However, the long-term genetic consequences of selfing are largely known. Mating system, which is responsible for gene transmission between plants and generations, is one of the most important factors affecting the

pattern of gene diversity of plant populations (Loveless and Hamrick 1984; Hamrick and Godt 1996; Glemin et al. 2006). Outcrossing is more advantageous than selfing, because it maintains higher levels of gene diversity in populations, which should increase their adaptive potential. In fact, selfing causes a reduction of gene diversity that has repeatedly been observed (Mabble et al. 2005; Charlesworth 2006). Moreover, the potential benefits of selfing may be counteracted by inbreeding depression, that is, fitness reduction of selfed progeny in relation to that of outcrossed progeny (Lande and Schemske 1985). In species with repeated selfing, purging effects may eventually lead to decreased inbreeding depression (Crnokrak and Barrett 2002); as a consequence, the numerical transmission advantage (3:2) of selfers can drive their increase in populations (Fisher 1941; Barrett 2010; Busch and Delph 2011). However, mixed-mating taxa can have inbreeding depression rates as great as those for outcrossing taxa, indicating that allele purging does not always occur (Winn et al. 2011). In some species with mixed-mating systems, high levels of inbreeding depression hinder the recruitment of selfed progeny, thus maintaining high genetic diversity in the population (Michalski and Durka 2007). However, in others, reproductive assurance benefits override the inbreeding depression detriment, and plants of selfed origin are maintained in populations despite their low genetic diversity (Michalski and Durka 2007; Arista et al. 2017). Thus, in conditions of pollen limitation, the reproductive assurance benefits of selfing could be selected (Schoen and Brown 1991; Cheptou and Massol 2009) and it can be an important mechanism in maintaining polymorphisms (Narbona et al. 2018) if inbreeding depression is not too high and selfing plants reach reproduction.

To the best of our knowledge, the role of selfing as a factor maintaining flower colour polymorphism has been described exclusively in *Ipomoea purpurea* (Fry and Rausher 1997; Rausher et al. 1993; Subramaniam and Rausher 2000). In this species, insects stop visiting a floral phenotype when its frequency is very low; these plants then produce seeds through automatic self-pollination, which increases their frequency in the next generation (Subramanian and Rausher 2000). However, given that more than 40% of species have mixed-mating systems (Goodwillie et al. 2005; Shivanna 2015) it is very likely that other polymorphic species show reproductive assurance, this being a neglected mechanism to maintain polymorphisms (Narbona et al. 2018). Nonetheless, consistent differences in mating systems between colour morphs should contribute to reproductive isolation and could initiate a speciation process (Nosil et al. 2009). Flower colour is considered a trait that promotes speciation by contributing to assortative mating between morphs (Servedio et al. 2011). Thus, colour polymorphism can promote the generation of new species; this process ends with the loss of that polymorphism (Hugall and Stuart-Fox 2012).

Here we study the role of selfing capacity in maintaining flower colour polymorphism in *Lysimachia arvensis*. This species has plants with red or blue flowers that show a geographical pattern of morph distribution; the blue morph is associated with the more stressed and dry zones of the Mediterranean Basin and the red with more temperate areas (Arista et al. 2013). In the Mediterranean Basin, most populations are monomorphic blue or blue-biased. Small solitary bees, the main pollinators of this species, discriminate clearly between flower morphs and show a strong preference for blue-flowered plants in Mediterranean populations (Ortiz et al. 2015); no information about pollinator attendance in non-Mediterranean areas exists. In the Mediterranean, directional selection driven by pollinators gives rise to higher male and female fitness of the blue morph relative to the red morph (Ortiz et al. 2015). Flowers of both morphs show lateral and vertical herkogamy, sequentially (Jiménez-López et al. 2019), but they differ in the level of each herkogamy type and can self-pollinate autonomously at different times in their lifespan. In the red morph, self-pollen deposition is possible at the end of the flower lifespan (delayed selfing), allowing reproductive assurance if outcross pollination fails (Jiménez-López unpub. data).

Despite the fact that both abiotic and biotic factors negatively select for the red morph in the Mediterranean, it frequently appears in a low proportion in these populations. This maintenance suggests the existence of a possible mechanism protecting the red morph. We hypothesize that selfing confers reproductive assurance to the red morph when pollinator attendance is low, thus maintaining it in Mediterranean polymorphic populations. If this happens, the genetic diversity of red-flowered plants in Mediterranean populations should be lower than that of blue-flowered plants, and inbreeding depression should be low enough to allow plant recruitment. Moreover, selfing reduces gene flow among individuals (Michalski and Durka 2007); hence, it could be an isolation mechanism leading to speciation (Martin and Willis 2007; Brys et al. 2013). If the red morph reproduces predominantly by selfing, this fact could contribute to its reproductive isolation and ecological divergence from the blue morph, and one could expect a genetic divergence of morphs as different evolutionary lineages, or even as incipient species.

MATERIAL AND METHODS

Study species

Lysimachia arvensis (L.) U. Manns & Anderb. (former *Anagallis arvensis* L.) is a self-compatible annual forb which offers only pollen as a reward for pollinators. It inhabits cultivated fields, wastelands and coastal sands (Ferguson 1972), and is native to Europe, Northern Africa and Western Asia; however, it is widely distributed over a large

part of the world (Pujadas 1997). In Mediterranean areas, populations in which the two morphs coexist are frequent, although the blue morph is usually found in a higher proportion (Arista et al. 2013). In contrast, in the Atlantic or temperate areas of Europe, monomorphic red populations are the norm.

Molecular analyses

AFLP and nuclear microsatellite markers were used to assess how genetic variation is structured among and within populations and floral colour morphs of *L. arvensis*. To this end, 20 natural populations were sampled (Appendix 1): 14 from the Mediterranean Basin and six from Non-Mediterranean areas. Of these, 11 were polymorphic and nine monomorphic (four blue and five red; Table 1). Six plants of each colour morph were sampled in polymorphic populations and ten in monomorphic populations; leaves of these plants were dried in silica gel and stored until molecular analyses were made. Total genomic DNA was extracted from dry leaf tissue with a plant extraction kit (Invisorb Vegetal DNA Kit HTS 96, Invitek, Berlin, Germany) following the supplier's instructions. The average DNA concentration was estimated photometrically using a NanoDrop DS-11 Spectrophotometer (DeNovix).

AFLP analyses were performed according to Vos et al. (1995) on 203 individuals, of which 10% were additionally replicated in order to exclude non-reproducible bands. Total genomic DNA was digested with the restriction enzymes EcoRI/MseI. After digestion, adaptors were ligated on both ends of genomic fragments and a two-step selective amplification was performed. We chose six selective primer pairs: EcoRI (Ned)-AAC/MseI-CTG, EcoRI (Vic)-ACG/MseI-CTG, EcoRI (Fam)-ACT/MseI CAG, EcoRI (Ned)-AGC/MseI CTC, EcoRI (Vic)-ACG/MseI-CAT, and EcoRI (Fam)-ACC/MseI-CTC. Resulting PCR products were separated by capillary gel electrophoresis on an automated sequencer (3730 DNA Analyser, PE Applied Biosystems, Foster City, CA, USA) with an internal size standard (GeneScan 500 LIZ, Applied Biosystems) at STABVIDA Lda. (Oeiras, Portugal). AFLP patterns were visualized with GeneMarker 1.9 (SoftGenetics, State College, PA, USA) for manual scoring of fragments after normalisation of the profiles. A fluorescence threshold set at 100 relative fluorescent units was applied to validate the peaks which exceeded the fluorescence intensity of this threshold. Amplified fragments from 100 to 500 base pairs were scored and exported as a presence/absence matrix.

The same 203 individuals were analysed at nine microsallite loci (*Lys*11, *Lys*12, *Lys*16, *Lys*28, *Lys*29, *Lys*30, *Lys*31, *Lys*32 and *Lys*33) previously characterized and available for *Lysimachia arvensis* (Jiménez-López et al., 2015). PCR products produced clear amplifications of the expected size on agarose gels. The amplification products were

Table 1. Gene diversity estimates for AFLP and mean of nine SSR microsatellites for each population and colour morph of *Lysimachia arvensis*. Measurements were taken in six red-flowered and six blue-flowered plants in polymorphic populations and in ten plants in monomorphic populations. Population details are in Appendix 1, available online. Region (M, Mediterranean; NM, Non-Mediterranean). Population type (P, polymorphic; M, monomorphic). HD, gene diversity; A, Allele number per locus; Ho, observed heterozygosity; He, expected heterozygosity; G_{IS}, inbreeding coefficient (**p<0.01; *p<0.05).

				AFLP	SSR			
Populations	**Region**	**Population type**	**Flower colour**	**HD**	**A**	**Ho**	**He**	**G_{IS}**
IT-Cer	M	P	Blue	0.118	4.000	0.477	0.666	0.064
IT-Cer	M	P	Red	0.074	2.889	0.111	0.430	0.325**
TR	M	P	Blue	0.085	3.333	0.457	0.588	0.222**
TR	M	P	Red	0.043	2.333	0.263	0.284	0.073
ES-Av	NM	P	Blue	0.049	2.556	0.470	0.463	-0.016
ES-Av	NM	P	Red	0.044	2.000	0.293	0.296	0.012
PR-Az-1	NM	P	Blue	0.044	2.444	0.296	0.342	0.133**
PR-Az-1	NM	P	Red	0.069	2.889	0.611	0.519	-0.177**
IT-Sc	M	P	Blue	0.081	3.333	0.567	0.623	0.091*
IT-Sc	M	P	Red	0.062	2.222	0.356	0.395	0.097
TN-1	M	P	Blue	0.132	3.667	0.604	0.655	0.079*
TN-1	M	P	Red	0.099	2.556	0.378	0.396	0.046
GR-Cr	M	P	Blue	0.114	3.556	0.614	0.595	-0.031
GR-Cr	M	P	Red	0.073	2.778	0.348	0.429	0.188*
MA-1	M	P	Blue	0.110	2.778	0.531	0.537	0.011
MA-1	M	P	Red	0.094	3.111	0.317	0.348	0.089**
ES-Ca-Zh	M	P	Blue	0.092	3.667	0.600	0.637	0.058
ES-Ca-Zh	M	P	Red	0.067	2.222	0.330	0.303	-0.089
TN-2	M	M	Blue	0.056	3.667	0.550	0.577	0.046
ES-Co1	M	M	Blue	0.070	4.000	0.531	0.630	0.156**
MA-2	M	M	Blue	0.065	3.778	0.550	0.612	0.101**
ES-Ma	M	M	Blue	0.081	4.000	0.538	0.638	0.156**
ES-Co2	M	M	Red	0.053	2.222	0.226	0.309	0.270**
ES-Ca-Gr	M	P	Blue	0.068	3.000	0.556	0.582	0.045
ES-Ca-Gr	M	P	Red	0.088	2.556	0.359	0.357	-0.007
ES-Te	NM	P	Blue	0.056	3.111	0.396	0.496	0.200**
ES-Te	NM	P	Red	0.074	3.667	0.678	0.638	-0.062
ES-Po	NM	M	Red	0.102	3.222	0.278	0.563	0.507**
PR-Az-2	NM	M	Red	0.079	3.111	0.485	0.470	-0.032
GR	NM	M	Red	0.093	3.444	0.381	0.531	0.281**
CH	M	M	Red	0.091	3.222	0.570	0.507	-0.126**

separated by capillary gel electrophoresis on an automated sequencer (3730 DNA Analyser, PE Applied Biosystems, Foster City, CA, USA) with an internal size standard (GeneScan 500 LIZ, Applied Biosystems) at STABVIDA Lda. (Oeiras, Portugal). The scoring was carried out with GeneMarker 1.9 (SoftGenetics, State College, PA, USA) following the same procedure as for AFLP markers.

Gene diversity was estimated separately with data from each molecular marker, for blue vs red morphs and for Mediterranean vs non-Mediterranean populations. From AFLP data, gene diversity was calculated as average gene diversity (HD) with GENEPOP v4.0.10 (Raymond and Rousset 2011). From SSR microsatellites data, gene diversity was calculated as expected heterozygosity (He) with GENODIVE, version 2.0b25 (Meirmans and Van Tienderen 2004), and observed heterozygosity (Ho) with ATETRA version 1.2 (Van Puyvelde et al. 2010). Allele number (A), and inbreeding coefficient (G_{IS}) were also calculated with GENODIVE 2.0b25, assuming infinite alleles and corrected for unknown allele dosage. Linkage disequilibrium (LD) was calculated with genetics R package (Warnes and Leisch 2005) after diploidization of each locus, and null allele frequency (No) was estimated by POLYSAT (Clark and Jasieniuk 2011). Voucher specimens were deposited at the Herbarium of the Seville University (SEV).

Inbreeding depression throughout life cycle

Inbreeding depression (ID) at different stages of the life cycle was studied for both colour morphs under natural Mediterranean field conditions. Seeds from plants of both colours were collected in the field, germinated and grown in glasshouses. For each separate colour morph, hand self- and cross-pollinations were carried out to obtain selfed and crossed seeds. The number of seeds per fruit after selfing and outcrossing was recorded in 40-110 fruits of each cross type and colour morph (hereafter seed production of mother plants).

A total of 1538 selfed and 1507 outcrossed seeds were sown in two natural Mediterranean populations: Dos Hermanas and Sevilla. 1366 seeds were from blue plants and 1679 from red ones. Both areas consist mainly of herbaceous communities on waste lands around orchards. In these populations, seeds were placed in individual cardboard pots and each potted seed was treated as an independent experimental unit. Sowing was carried out at the beginning of November, and potted plants were harvested at the end of May. During the growth cycle pots were checked every fortnight, and time from sowing to germination (hereafter time to germination), seed germination, seedling survival up to reproductive age, time from germination to flowering (hereafter time to flowering) and seed production after free pollination

(hereafter seed production of progeny) were recorded for each plant. Seed production of progeny was estimated as the mean number of seeds in two ripe fruits per plant.

Partial ID coefficients (δ_i) were calculated for each colour morph and population at each of the following life stages: seed production of mother plants (δ_{sm}), total seed viability (δ_{tv}), seedling survival (δ_{ss}), and seed production of progeny (δ_{sp}). To avoid bias in the assessment of inbreeding depression from germination data due to seed dormancy, a subset of selfed and outcrossed seeds was sown in Petri dishes and placed in a germination chamber. Non-germinated seeds (204 self red, 198 cross red, 168 self blue and 168 cross blue) were placed in a 100μl solution of tetrazolium 0.11% to determine their viability (Glenner 1990). Data regarding germination and viability of non-germinated seeds was then considered together to calculate inbreeding depression at that stage (total seed viability, δ_{tv}). Partial ID coefficients were calculated using the expression proposed by Ågren and Schemske (1993):

$$\delta_i = (W_{io} - W_{is})/W_{imax}$$

where $\boldsymbol{\delta_i}$ is the ID coefficient at life-stage **i**, $\mathbf{W_{io}}$ is fitness after outcrossing, $\mathbf{W_{is}}$ is fitness at this life-stage after selfing, and $\mathbf{W_{imax}}$ is the maximum fitness at this stage (W_{io} or W_{is}). If $W_{io}>W_{is}$, δ values are positive and inbreeding depression exists at this stage, while in $W_{is}>W_{io}$, δ values are negative and outbreeding depression may occur. Cumulative ID coefficients (δ_T) were also calculated for each colour morph and population. Values of fitness at each life-stage were relativised to the maximum at this stage; cumulative fitness (W_T) for each cross type ($W_{To}$and W_{Ts}) was then estimated by multiplying relative fitness values at the four life-stages considered (seed production of mother plants, total seed viability, seedling survival and seed production of progeny). In this way, cumulative fitness is presented as a proportion. Then, as for partial coefficients, δ_T was calculated by using the expression: $\delta_T = (W_{To}-W_{Ts})/W_{Tmax}$.

Statistical analyses

To apportion genetic variation as estimated from both molecular markers among and within populations and floral colour morphs, multi-locus analyses of molecular variance (AMOVA) were performed using Arlequin (Schneider et al. 2000). These analyses hierarchically partitioned molecular variation into within- and among-population components to estimate genetic structure in the following predefined groups: blue vs red plants, Mediterranean vs non-Mediterranean, blue Mediterranean vs red Mediterranean, blue non-Mediterranean vs red non-Mediterranean. Permutation tests were used to determine statistical significance (Excoffier et al. 1992). For SSR, statistics for the significance (OSx-statistic, Goudet 1995) across all groups or between pairs of comparison were obtained by 9999 randomizations using GENODIVE 2.0b25.

In exploring the possibility of inbreeding depression, differences in seed production of mother plants and viability of non-germinated seeds were analysed with colour and cross-type (selfing or outcrossing) as fixed factors and taking their interaction into consideration. In analysing differences in time to germination, seed germination, seedling survival, time to flowering and seed production of progeny, the factors population, cross type and colour were considered fixed, and three-way interaction was also considered. GLMs were carried out with different link functions and error distributions, depending on the type of response variable modelled. Binomial distribution of errors and logit link function were used to analyse germination, viability and survival. Binomial negative distribution with log link function was used to analyse time to germination, and normal distribution to analyse time to flowering and seed production. All these analyses were carried out using the GLM module of SPSS (IBM SPSS Statistic 23, 2015, USA) with Type III tests. When GLMs showed significant differences, the means of treatment were compared using t-tests based on standard errors calculated from the specific model.

RESULTS

Properties of AFLPS and microsatellites

The total number of AFLP bands found among populations of *L. arvensis* was 870, of which 82.9% were polymorphic. All individuals had unique AFLP phenotypes and the reproducibility of the AFLP bands was 10%. The nine microsatellite loci were successfully genotyped in the 203 individuals of *L. arvensis*. There were cases of deviation from HWE ($P < 0.05$) after Bonferroni correction across populations and loci; the most significant cases were related to negative or high levels of G_{IS}, indicating HWE deviation caused by heterozygote excess. Null allele frequency (No) estimated using POLYSAT resulted in the highest frequency of 0.478 (IT-Sc-B/Lys12), with an average frequency of 0.138 over all of the nine markers. Significant LD was not found between any pairwise combinations of loci ($p < 0.05$) after Bonferroni correction.

Gene diversity and population structure

In a total of 203 plants and nine SSR analysed, the total number of alleles was 74 and the frequency of alleles per locus by population and morph ranged from 1.602-2.925. Gene diversity estimates varied between markers; the lower values were found in AFLP (HD) ranging from 0.043-0.118 (mean 0.078 ± SD 0.022) while for multi-locus SSR data, expected heterozygosity (He) ranged from 0.283-0.666 (average 0.497 ± SD 0.027; Table 1, Table 6_suppInfo). Per locus, He ranged from 0.000-0.862, observed heterozygosity (Ho) from 0.000-0.900 and the inbreeding coefficient (G_{IS})

from -0.800 to 1.000. Positive values of G_{IS} were found in the nine loci, indicating heterozygote deficiency (Table 1, Table 6_suppInfo).

For AFLP, blue plants showed higher gene diversity (0.080 ± 0.006) than red ones (0.065 ± 0.006). For SSR, the blue morph showed significantly higher observed heterozygosity (Ho) values and lower inbreeding coefficient (G_{IS}) than the red morph (Ho: 0.562 blue vs 0.415 red, G_{IS}: 0.330 blue vs 0.618 red; $p<0.05$ in all cases). However, the expected heterozygosity was similar between morphs (He: 0.657 blue vs 0.650 red; $p>0.05$). Taking areas into account, blue plants showed higher gene diversity for AFLP (0.093 ± 0.006) than red ones (0.065 ± 0.006) in Mediterranean areas, but the opposite pattern was observed in non-Mediterranean populations (blue 0.043 ± 0.011; red 0.064 ± 0.008). For SSR, the blue morph showed significantly higher observed and expected heterozygosity and lower inbreeding coefficient than the red morph in Mediterranean areas; in contrast, in non-Mediterranean areas the observed and expected heterozygosity were statistically similar between morphs, but the inbreeding coefficient was statistically higher in the blue morph (Table 2).

Table 2. Mean values (standard error) of gene parameters for blue and red morphs of *L. arvensis* at nine SSR loci in Mediterranean and non-Mediterranean populations. Within each column, means followed by the same letter are statistically similar. * $p<0.05$

Type of population	Colour morph	Observed Heterozygosity (Ho)	Expected Heterozygosity (He)	No. of alleles per locus	Inbreeding Coefficient (Gis)
Mediterranean	Blue	0.560 (0.039)a	0.611 (0.030)a	6.33 (0.745)a	0.116* (0.034)a
	Red	0.327 (0.066)b	0.381 (0.054)b	6.00 (0.866)a	0.204* (0.057)b
Non-Mediterranean	Blue	0.388 (0.073)c	0.434 (0.045)c	4.44 (0.503)b	0.141* (0.043)ab
	Red	0.486 (0.055)c	0.499 (0.036)c	5.78 (0.722)ab	0.086* (0.040)c

AMOVA consistently demonstrated a significant population structure separating blue plants from red at both AFLP and SSR markers (Table 3). Mediterranean populations were also differentiated from non-Mediterranean at both markers, although the proportion of explained variance was low. Taking into account flower colour, there was significant differentiation between blue and red Mediterranean plants at both SSR and AFLP markers; 59.59% of variance at SSR and 25.31% at AFLP were due to differences between colours. In non-Mediterranean areas blue and red plants also showed genetic differentiation, the variance attributed to flower colour at SSR and AFLP markers being 52.37% and 36.50%, respectively (Table 3).

Table 3. Results of analyses of molecular variance (AMOVA) for AFLP and SSR markers and for different groupings of populations. For microsatellites a total of 9 loci were used. Statistical significance is based on 10100 permutations. * p<0.05, ** p<0.01, ***p<0.001.

	Source of variation	d.f	Sum of squares	Variance components	% Variance explained	Fixation Indices	P
Blue vs Red							
Microsatellites	Among groups	1	628.363	5.99817	57.19221	FSC: 0.66957	***
	Among populations within groups	29	608.400	3.00610	28.66296	FST: 0.85855	***
	Within populations	172	255.158	1.48347	14.14483	FCT: 0.57192	***
AFLP	Among groups	1	3549.297	30.77457	32.54	FSC: 0.45886	***
	Among populations within groups	29	6805.726	29.26906	30.95	FST: 0.63497	***
	Within populations	183	6316.739	34.51770	36.50	FCT: 0.32545	***
Mediterraneanvs Non-Mediterranean							
Microsatellites	Among groups	1	66.654	0.29612	3.81415	FSC: 0.80135	***
	Among populations within groups	29	1170.109	5.98413	77.07807	FST: 0.80892	***
	Within populations	172	255.158	1.48347	19.10777	FCT: 0.03814	*
AFLP	Among groups	1	858.094	5.27732	6.38	FSC: 0.55408	***
	Among populations within groups	29	9496.929	42.89033	51.87	FST: 0.58254	***
	Within populations	183	6316.739	34.51770	41.75	FCT: 0.06382	*
Blue MediterraneanvsRed Mediterranean							
Microsatellites	Among groups	1	516.094	6.45105	59.59842	FSC: 0.63192	***
	Among populations within groups	20	414.295	2.76347	25.53052	FST: 0.85129	***
	Within populations	121	209.258	1.60967	14.87107	FCT: 0.59598	***
AFLP	Among groups	1	1834.079	22.93984	25.31	FSC:0.47766	***
	Among populations within groups	20	5045.164	32.33852	35.68	FST: 0.60986	***
	Within populations	127	491.133	35.36325	39.01	FCT: 0.25308	***
BlueNmedvsRedNmed							
Microsatellites	Among groups	1	116.934	4.64348	52.37656	FSC:0.74116	***
	Among populations within groups	7	122.786	3.12923	35.29645	FST: 0.87673	***
	Within populations	51	45.900	1.09286	12.32698	FCT:0.52377	***
AFLP	Among groups	1	1060.195	33.96240	36.50	FSC: 0.44836	***
	Among populations within groups	7	1557.491	26.49618	28.47	FST: 0.64968	***
	Within populations	56	1825.606	32.60011	35.03	FCT:	**

Inbreeding depression throughout life-cycle

The number of seeds per capsule produced after hand-pollination of mother plants differed between treatments (self and cross) and colours (Table 4). In general, seed

Table 4. Summary of GLM results for different effects: population (Seville/Dos Hermanas), treatment (selfing/outcrossing) and colour morph (blue/red) and their interactions, on different traits measured in *Lysimachia arvensis*. Significant values in bold.

Dependent variable	Effects	Wald chi-square	Df	*P*
Seed production of mother plants	Treatment	16.211	1	0.000
	Colour	20.335	1	0.000
	T x C	9.232	1	0.002
Time to germination	Population	18.097	1	0.000
	Treatment	12.31	1	0.000
	Colour	18.91	1	0.000
	P x T x C	7.85	4	0.097
Germination	Population	11.631	1	0.001
	Treatment	16.286	1	0.000
	Colour	26.709	1	0.000
	P x T x C	90.022	4	0.000
Viability of non-germinated seeds	Treatment	191.231	1	0.000
	Colour	0.884	1	0.347
	T x C	1.003	1	0.317
Seedling survival	Population	1.61	1	0.204
	Treatment	2.06	1	0.151
	Colour	34.58	1	0.000
	P x T x C	24.32	4	0.000
Time to flowering	Population	89.42	1	0.000
	Treatment	286.60	1	0.000
	Colour	170.93	1	0.000
	P x T x C	46.88	4	0.000
Seed production of progeny	Population	0.70	1	0.400
	Treatment	52.48	1	0.000
	Colour	76.74	1	0.000
	P x T x C	8.16	4	0.080

production was higher in the red morph than in the blue one, and it was also higher after selfing than after outcrossing (Fig. 1). However, the colour-by-treatment interaction was significant (Table 4), as only in the blue morph was seed production significantly higher after selfing than after outcrossing (Fig. 1). Thus, at this first stage of the life cycle, ID coefficient was negative for both colour morphs, although it was very close to zero for the red morph (Table 5).

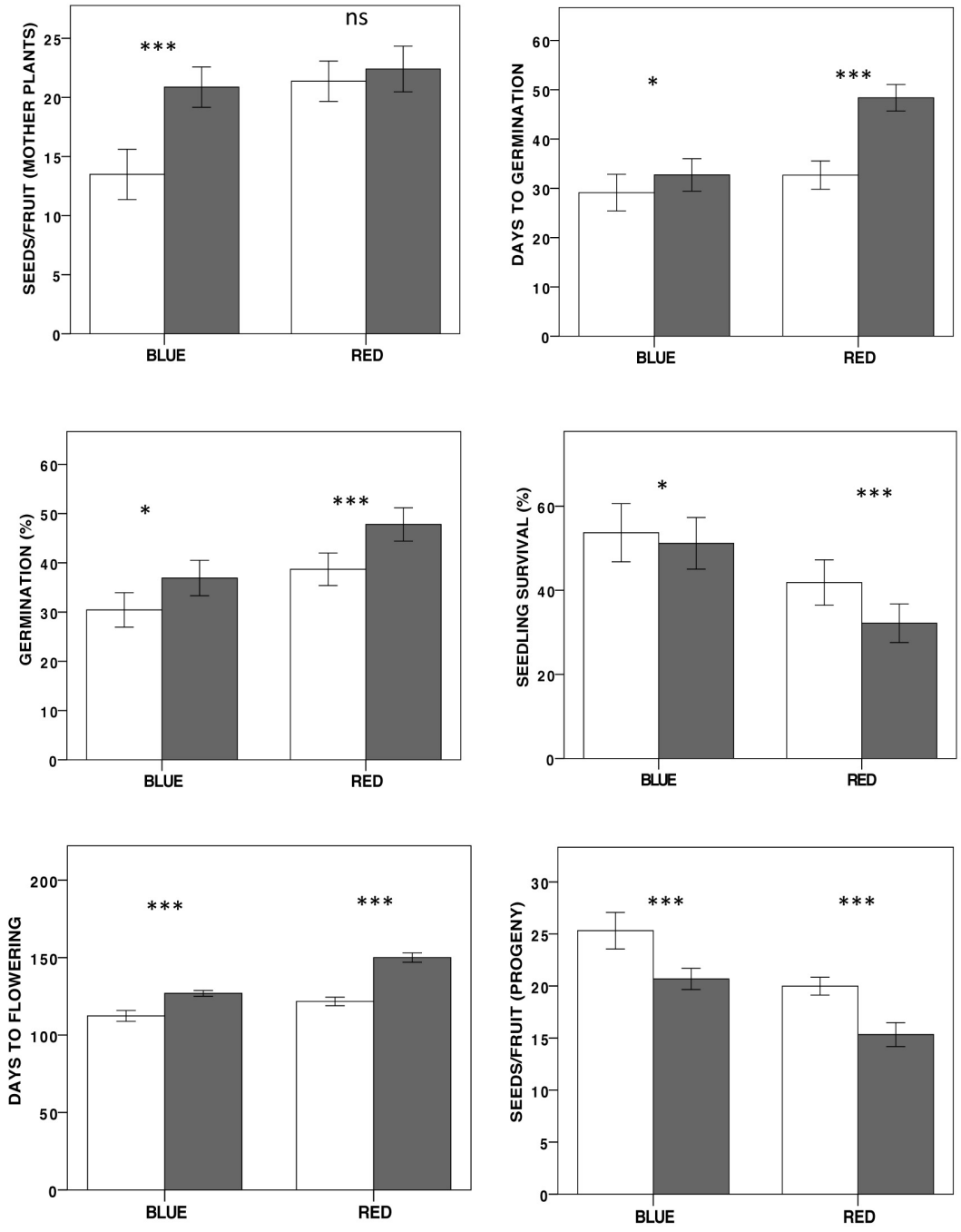

Figure 1. Differences in whole-life fitness components between selfed and outcrossed progeny from the blue and red morphs of *Lysimachia arvensis* in the Mediterranean. Means ±SE are shown. In each graph, asterisks indicate significant differences between selfing and outcrossing values for each colour morph. ***, $p<0.001$, **, $p<0.01$, *, $p<0.05$, ns. Not significant.

Table 5. Estimates of inbreeding depression for the two colour morphs of *Lysimachia arvensis* in two populations. Wo and Ws are fitness measures after outcrossing and selfing, respectively, at different life stages (seed production of mother plants, number per fruit; total seed viability, %; seedling survival, %; and seed production of progeny, number per fruit) or total for the life cycle (cumulative, proportion); δi is the inbreeding depression coefficient at each of the stages or its cumulative value.

	Seed production of mother plants		**Total seed viability**		**Seedling survival**		**Seed production of progeny**		**Cumulative**	
	Blue	**Red**	**Blue**	**Red**	**Blue**	**Red**	**Blue**	**Red**	**Blue**	**Red**
Sevilla										
Wo	13.49	21.37	85.12	89.12	46	35	23.84	21.48	0.44	0.95
Ws	20.87	22.40	32.85	57.50	67	28	22.54	15.37	0.36	0.37
δi	-0.35	-0.05	0.61	0.35	-0.31	0.20	0.05	0.28	0.18	0.61
Dos Hermanas										
Wo	13.49	21.37	82.00	90.14	62	49	25.31	19.98	0.65	0.95
Ws	20.87	22.40	62.60	54.10	44	36	20.68	15.33	0.44	0.34
δi	-0.35	-0.05	0.24	0.40	0.29	0.27	0.18	0.23	0.32	0.65

In the year studied, two rainy periods occurred and seed germination took place mainly thereafter (Fig. 2). Germination response differed between treatments, colours and populations, but three-way interaction was not significant (Table 4). In general, seeds germinated earlier in the Seville population (30 vs. 39 days), and outcrossed seeds germinated earlier in both populations (31 vs. 38 days; Fig. 2). Moreover, seeds from the blue morph germinated more quickly than those from the red morph (mean 29 vs. 39 days respectively).

The percentage of seed germination also differed between treatments, colour and populations (Table 4). In general, germination was higher in Dos Hermanas than in Seville, in red plants than in blue ones, and in selfed than in outcrossed seeds. However, three-way interaction was significant as the germination patterns were not congruent among populations. Seed viability of non-germinated seeds only differed between treatments (Table 4); most non-germinated outcrossed seeds were dormant but viable, while non-germinated selfed seeds were mainly unviable. Taking into account germination and viability of non-germinated seeds, ID coefficient at this stage was high, although it showed contrasting patterns between morphs and populations (Table 5).

Seedling survival showed differences between colours but not between populations or treatments (Table 4). Three-way interaction was significant here, as a different pattern of survival was shown in each population (Fig. 2). ID coefficient was negative for blue plants in Seville but positive for the remaining cases, ranging from 0.20-0.29 (Table 5).

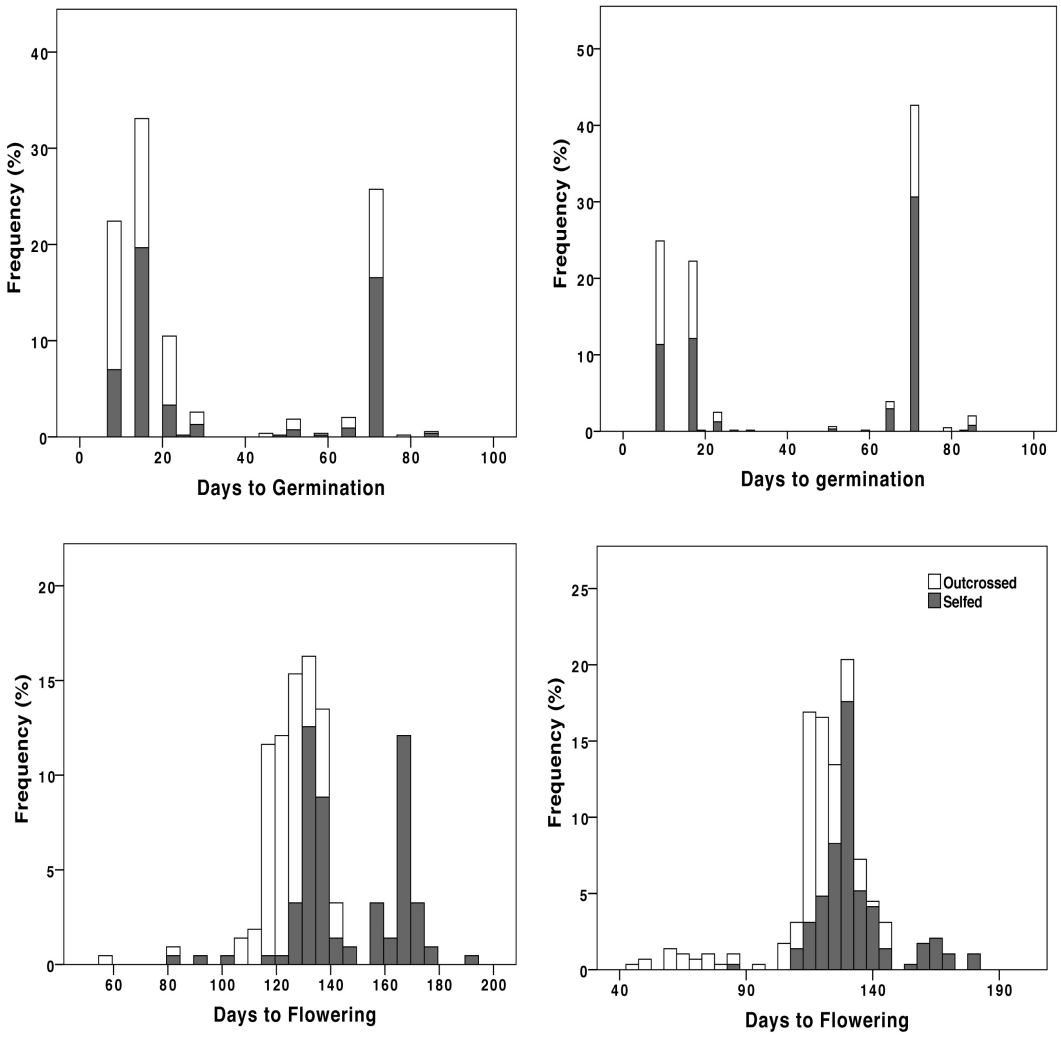

Figure 2. Frequency distributions for two life cycle traits: time to germination and time to flowering of selfed and outcrossed progeny of *Lysimachia arvensis* in two natural populations.

Flowering time showed significant differences between populations, treatments and colour morphs (Table 4), the flowering order being as follows: blue outcrossed plants, blue selfed plants, red outcrossed plants and red selfed plants (Fig. 2). These flowering orders appeared in the two populations studied and as a consequence, there were *L. arvensis* plants in flower for almost five months.

In free pollination, the number of seeds per fruit varied between treatments and colour morphs, but not between populations (Table 4). Outcrossed plants produced a mean of 22.6 seeds while selfed plants produced 18.5 seeds. Blue morphs also produced a higher number of seeds per fruit than red morphs (23.09 vs. 10.04); these differences were found in both populations.

Cumulative inbreeding depression measures were positive for both morphs in both populations, and were higher for the red than for the blue morph. The red morph showed a consistent high ID value of around 0.6 in both populations, whereas in the blue morph it varied from 0.19 in Seville to 0.36 in Dos Hermanas (Table 5).

DISCUSSION

Three main results are derived from this study: 1) a high genetic differentiation between colour morphs that strongly suggests reproductive isolation, 2) a lower genetic diversity of the red morph relative to the blue morph, mainly in Mediterranean areas, which suggests differences in breeding system between morphs and 3) a higher inbreeding depression rate for the red morph relative to the blue morph that would render recruitment of selfed progeny difficult in the populations.

Fitness differences of selfing and outcrossing progeny

Marked differences in the fitness of the progeny derived from selfing and outcrossing were found in both colour morphs throughout their life cycle. Seed production from selfing originated a higher number of seeds than that from outcrossing in both morphs. This result was unexpected, given that a high impact of selfing was found in the remaining steps in the life cycle; also, seed viability was much higher after outcrossing than after selfing. Given that the flowers of *L. arvensis* are very small and that emasculation is difficult without damaging the flower, floral manipulation should be considered as a possible cause of decreased seed production in hand-outcrossed flowers. Seed germination was also higher in selfed seeds, but this was merely a consequence of differences in seed dormancy between selfed and outcrossed seeds. All outcrossed seeds that did not germinate were viable, while ungerminated selfed seeds were dead. Given differences in germination between morphs, this means that seed-banks of *L. Arvensis* in the wild should consist of outcrossed seeds from which individuals could be incorporated into the populations every year.

The progeny derived from selfing also showed marked differences in phenology in relation to that derived from outcrossing in both morphs. This was an unexpected finding, because differences in phenology according to breeding system are not usually reported in the literature. Non-dormant seeds of *L. arvensis* germinated just after a rainy period giving rise to pulses in which the germination order was: first outcrossed blue, then selfed blue and outcrossed red, and selfed red last. In annuals, the time of germination is the first major developmental transition influencing all posterior life cycle traits (Finch-Savage and Leubner-Metzger 2006; Manzano-Piedras et al. 2014). Arid

environments such as those in the Mediterranean are characterized by limited and variable rainfall that supplies resources in pulses (Chesson et al. 2004). In these environments, quick germination just after rain permits seedlings to develop deep roots to tolerate water scarcity, thereby increasing survival probability (Schenk and Jackson 2002). As blue seeds germinated earlier than red ones, differences in survival found between colours could be a result of germination differences. Survival differences between morphs in dry environments were found experimentally in a previous work (Arista et al. 2013); thus this study confirms previous findings. In the red morph, differences in the time of germination between selfed and outcrossed seeds were much more marked than in the blue morph; consequently, differences in survival between selfed and outcrossed seedlings were high.

Flowering phenology was also markedly affected by progeny origin, with blue plants flowering earlier than red ones, and with outcrossed plants flowering earlier than selfed ones. This implies a variation in mating phenology between colour morphs, as was already reported in the greenhouse (Arista el al. 2013) although overlap also occurs. Differences in reproductive timing often have strong fitness consequences, as they promote assortative mating (e.g. Fox and Kelly 1993; Fox 2003). Differences in flowering phenology between morphs could limit pollen flow between them in polymorphic populations, as assortative mating within colours is frequent. Even if colour morphs show a temporal overlap in flowering phenology, assortative mating can be much stronger than expected as the chance of mating is reduced (Fox 2003). Thus, a difference in flowering phenology acts as a prezygotic barrier to gene flow (Martin and Willis 2007; Botes et al. 2008) and given that prezygotic barriers generally make a greater contribution to reproductive isolation than postzygotic barriers (Lowry et al. 2008; Widmer et al. 2009), mating phenology could contribute efficiently to morph isolation in *L. arvensis*. On the other hand, flowering phenology is constrained by both abiotic and biotic factors (Cruz-Neto et al. 2011), and can strongly influence plant reproductive success (Ollerton and Lack 1998). In annual plants, an early flowering when water is available permits an extended flowering period, and in seasonal climates such as the Mediterranean, this is advantageous as it assures plant reproduction (Rodriguez-Pérez and Traveset 2016). In contrast, a late flowering increases the risk of drought and limits vegetative growth and fruit production (Gimenez-Benavides et al. 2007). In fact, we have found that seed production showed the same pattern as flowering phenology, with higher production in plants flowering earlier (outcrossed blue) and lower in plants flowering later (selfed red). Thus, in the Mediterranean populations studied, the very late flowering of selfed red plants is markedly disadvantageous, strongly limiting seed production. However, given that the plants studied grew in natural areas, differences in seed production between morphs could also be a

consequence of differential pollinator attendance to morphs, as reported in some natural Mediterranean populations of *L. arvensis* (Ortiz et al. 2015).

Genetic variation between colour morphs

The analyses of genetic variation in *L. arvensis* at both neutral DNA markers showed a strong partitioning of molecular variation between the red and blue morphs in both Mediterranean and non-Mediterranean areas. Differences between colour morphs explained most variations in both AFLP and SSR markers. Genetic differentiation between colour morphs in the populations studied strongly indicates that gene flow between them is restricted, morphs being to some extent reproductively isolated. This result is supported by two facts: first, differences in flowering phenology found here and in a previous study (Arista et al. 2013) that hinder pollen flow between morphs, and second, differences in pollinator attendance in polymorphic populations where pollinators prefer blue flowered plants and show floral constancy (Ortiz et al. 2015; Jiménez-López et al. unpub. results). In addition, the subtle but consistent differences in herkogamy traits found between morphs (Jiménez et al. unpub. data) could also be a consequence of evolutionary divergence owing to morph isolation.

The red morph had a consistently lower genetic diversity, lower observed heterozygosity and lower number of alleles than the blue morph. Plant mating systems have significant effects on genetic diversity (reviewed by Charlesworth and Wright 2001), with selfers showing much lower diversity than outcrossers. These differences between selfers and outcrossers are expected to be even more pronounced when both kinds of plants co-occur within populations (Glémin et al. 2006). Thus, the set of differences in gene diversity found between morphs suggests a higher selfing rate in the red morph. The strongest differences in gene diversity were found in Mediterranean areas, where moreover the blue morph showed a higher diversity and a lower inbreeding coefficient. In contrast, in non-Mediterranean areas results for the two markers were not congruent; red plants showed higher genetic diversity than blue ones when AFLP was used, but both morphs showed similar diversity with SSR microsatellites. In non-Mediterranean areas, the inbreeding coefficient showed the opposite pattern to that found in Mediterranean areas, since it was higher for the blue morph, although similar to that in the Mediterranean. Differences in pollinator attendance in Mediterranean and non-Mediterranean areas could explain these differences. In a previous study, pollinators showed a marked preference for the blue morph in blue-biased populations and a similar preference for both morphs in balanced populations (Ortiz et al. 2015). The authors explained that result as a consequence of positive frequency-dependent pollinator attendance, a very common situation in natural populations (Cresswell and Galen 1991; Smithson and MacNair 1996). Given that the

blue morph is more frequent in Mediterranean areas while the red morph is much more frequent in non-Mediterranean areas (Arista et al. 2013), differential attendance of pollinators could explain differences in inbreeding coefficient between morphs. This would imply that mating system is context-dependent for the red morph, being mainly selfing in the Mediterranean but outcrossing in non-Mediterranean areas. In contrast, the similar inbreeding coefficient of the blue morph in both areas suggests a similar breeding system in both Mediterranean and non-Mediterranean areas, although parameters of genetic diversity were higher in the Mediterranean.

The higher inbreeding coefficient and the low genetic diversity of red plants in the Mediterranean suggest that some selfed progeny is recruited in populations, despite the high values of inbreeding depression throughout the life cycle found in the field. Differences between selfed and outcrossed progeny were found in both morphs, although they varied in intensity. The ranges of inbreeding depression found in *L. arvensis* are in accordance with those of plants with mixed reproductive systems, ranging from 0.2 to 0.8 (Winn et al. 2011). Interestingly, ID in the blue morph was close to that of selfing species (near of 0.2), while red morph ID was close to that of outcrossing (about 0.6). According to Winn et al. (2011) if purging is occurring, ID of mixed-mating species should be closer to that of selfing species as occurs in the blue morph; this suggests an evolutionary trend towards selfing. In contrast, the high ID of the red morph would suggest the existence of a mechanism to avoid purging, limiting evolution towards selfing and maintaining stability of the mixed-mating system in the red morph (Winn et al. 2011).

The high ID rates in the red morph in the two populations studied (about 0.6) mean that only 40% of selfed progeny could be recruited in populations, while outcross progeny recruits 100%. The impact of ID on the fitness of the red morph was markedly high, but selfing could be the sole way to ensure reproduction under pollen limitation. Importantly, annual plants must produce seeds before dying, and even low quality offspring make some contribution to fitness, defined as the representation of an individual's genes in progeny generation (Charlesworth 2006). Moreover, the delayed selfing mechanism of the red *L. arvensis* avoids an extra cost of pollen and seed discounting; consequently, any surviving selfed progeny would provide reproductive assurance (Kalisz et al. 2004) by maintaining the red morph. Given that the red morph is negatively selected by both biotic and abiotic factors in the Mediterranean Basin (Arista et al. 2013; Ortiz et al. 2015), the survival of any selfed progeny can help its maintenance in populations. The high ID limits the numerical advantage of selfing (3:2; revised in Jain 1976) and impedes an increase in the frequency of the red morph in Mediterranean polymorphic populations.

In short, we have found marked differences in the progeny coming from selfing and outcrossing in both morphs, but also between morphs. The earlier germination and flowering of outcrossed relative to selfed progeny, and that of the blue morph relative to the red morph, can represent an important recruitment limitation for red-flowered plants in Mediterranean areas. The low genetic diversity of the red morph is in accordance with a reproductive system based on predominant selfing. Despite this, the red morph showed higher inbreeding depression rates relative to those of the blue one, suggesting a limited capacity for recruitment of the selfed progeny of the red morph. The strong and consistent genetic differentiation between colour morphs in the populations studied indicates a history of gene flow limitation between them. Phenological and genetic differences between morphs found in this study add up to subtle morphological differences in herkogamy traits (Jiménez-López et al., unpub. data), strongly suggesting that the two colour morphs of *Lysimachia arvensis* are, in fact, different lineages.

REFERENCES

Ågren J. and D.W. Schemske. 1993. Outcrossing rate and inbreeding depression in two annual monoecious herbs, Begonia hirsuta and B. semiovata. *Evolution*, **47**: 125-135.

Arista M., M. Talavera, R. Berjano and P.L. Ortiz. 2013. Abiotic factors may explain the geographical distribution of flower colour morphs and the maintenance of colour polymorphism in the scarlet pimpernel. *J. Ecol.*, **101**, 1613–1622. doi: 10.1111/1365-2745.12151

Arista M., R. Berjano, J. Viruel, M.A. Ortiz, M. Talavera, and P.L. Ortiz. 2017. Uncertain pollination environment promotes the evolution of a stable mixed reproductive system in the self-incompatible *Hypochaeris salzmanniana* (Asteraceae). *Ann. Bot.*, **120**: 447-456.

Barrett S.C. 2010. Understanding plant reproductive diversity. *Philos. Trans. R. Soc. B-Biol. Sci. B,***365**: 99-109.

Botes C., S.D. Johnson, and R.M. Cowling. 2008. Coexistence of succulent tree aloes: partitioning of bird pollinators by floral traits and flowering phenology. *Oikos*, **117**: 875–882. doi: 10.1111/j.2008.0030-1299.16391.x

Brys R., A.V. Broeck, J. Mergeay and H. Jacquemyn. 2013. The contribution of mating system variation to reproductive isolation in two closely related *Centaurium* species (Gentianaceae) with a generalized flower morphology. *Evolution*, **68**: 1281–1293. doi: 10.1111/evo.12345.

Busch J.W. and L.F. Delph. 2011. The relative importance of reproductive assurance and automatic selection as hypotheses for the evolution of self-fertilization. *Ann. Bot.*, **109**: 553-562.

Campbell, D. R., N. M. Waser, and E. J. Meléndez-Ackerman. 1997. Analyzing pollinator-mediated selection in a plant hybrid zone: hummingbird visitation patterns in three spatial scales. *Am. Nat.,***149**: 245–315.

Charlesworth D. 2006. Evolution of Plant Breeding Systems. *Curr. Biol.*, **16**: R726–R735. doi: 10.1016/j.cub.2006.07.068

Charlesworth D. and S.I. Wright. 2001. Breeding systems and genome evolution. *Curr. Opin. Genet. Dev.*, **11**: 685–690. doi: 10.1016/S0959-437X(00)00254-9

Cheptou P.O. and F. Massol. 2009. Pollination fluctuations drive evolutionary syndromes linking dispersal and mating system. *Am. Nat.*, **174**: 46–55. doi: 10.1086/599303

Chesson P., R.L.E. Gebauer, S. Schwinning, N. Huntly, K. Wiegand, M.S.K. Ernest, A. Sher, A. Novoplansky and J.F. Weltzin. 2004. Resource pulses, species interactions, and diversity maintenance in arid and semi-arid environments. *Oecologia*, **141**: 236-253. doi: 10.1007/s00442-004-1551-1

Clark L. and M. Jasieniuk. 2011. Polysat: an R package for polyploid microsatellite analysis. *Mol. Ecol. Resour.*, **11**: 562–566. doi: 10.1111/j.1755-0998.2011.02985.x.

Cresswell J.E. and C. Galen. 1991. Frequency-dependent selection and adaptive surfaces for floral character combinations: the pollination of *Polemonium viscosum*. *Am*. *Nat*. **138**, 1342–1353.

Crnokrak P. and S.C.H. Barrett. 2002. Perspective: purging the genetic load: a review of the experimental evidence. *Evolution*, **56**: 2347–2358. doi: 10.1554/0014-3820(2002)056[2347:PPTGLA]2.0.CO;2

Cruz-Neto O., I.C. Machado, J.A. Duarte and A.V. Lopes. 2011. Synchronous phenology of hawkmoths (Sphingidae) and *Inga* species (Fabaceae-Mimosoideae): implications for the restoration of the Atlantic forest of northeastern Brazil. *Biodivers. Conserv.*, **20**: 751–765. doi: 10.1007/s10531-010-9975-x

Excoffier L., P.E. Smouseand J.M. Quattro. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, **131**: 479-491.

Fenster C.B., W.S. Armbruster, P. Wilson, M.R. Dudash and J.D. Thomson. 2004. Pollination syndromes and floral specialization. *Annu. Rev. Ecol. Evol. Syst*., **35**: 375–403. doi: 10.1146/annurev.ecolsys.34.011802.132347

Ferguson L.F. 1972. *Anagallis* L. In Tutin T.G. et al. (Eds.) *Flora Europaea*. Cambridge University Press. Cambridge, UK.

Finch-Savage W.E. and G. Leubner-Metzger. 2006. Seed dormancy and the control of germination. *New Phytol.*, **171**: 501–523. doi: 10.1111/j.1469-8137.2006.01787.x

Fisher R.A. 1941. Average excess and average effect of a gene substitution. *Annals of Eugenics*, **11**: 53-63.

Fox G.A. 2003. Assortative mating and plant phenology: evolutionary and practical consequences. *Evol. Ecol. Res*., **5**: 1-18.

Fox G.A. and C.K. Kelly. 1993. Plant phenology: neutrality and selection. *Trends Ecol. Evol.*, **8**: 34–35.

Fry J.D. and M.D. Rausher. 1997. Selection on a floral color polymorphism in the tall morning glory (*Ipomoea purpurea*): transmission success of the alleles through pollen. *Evolution*, **51**: 66-78.

Gigord L.D., M.R. Macnair and A. Smithson. 2001. Negative frequency-dependent selection maintains a dramatic flower color polymorphism in the reward less orchid *Dactylorhiza sambucina* (L.) Soo. *PNAS*, **98**: 6253–6255. doi: 10.1073/pnas.111162598

Giménez-Benavides L., A. Escudero and J.M. Iriondo. 2007. Reproductive limits of a late-flowering high-mountain Mediterranean plant along an elevational climate gradient. *New Phytol.*, **173**: 367–382. doi: 10.1111/j.1469-8137.2006.01932.x

Glémin S., E. Bazin and D. Charlesworth. 2006. Impact of mating systems on patterns of sequence polymorphism in flowering plants. *Proc. R. Soc. Lond. Ser. B-Biol. Sci.*, **273**: 3011-3019.

Glenner G.G.1990. Formazans and Tetrazolium Salts. In: Biological Stains. Edited by R.D. Lillie, MD Sigma Chemical Company. ISBN 0-941633-29-2. 225-235.

Goodwillie C., S. Kalisz and C.G. Eckert. 2005. The evolutionary enigma of mixed mating systems in plants: occurrence, theoretical explanations, and empirical evidence. *Annu. Rev. Ecol. Evol. Syst.*, **36**: 47-79.

Goudet J. 1995. FSTAT (version 1.2): a computer program to calculate F-statistics. *J. Hered.,* **86**: 485-486.

Hamrick J.L. and M.J.W. Godt. 1996. Effects of life-history traits on genetic diversity in plant species. *Philos. Trans. R. Soc. Lond. B*, **351**: 1291–1298. doi: 10.1098/rstb.1996.0112

Holsinger K.E. 2000. Reproductive systems and evolution in vascular plants. *Proc. Natl. Acad. Sci.*, **97**: 7037-7042.

Hugall A.F. and D. Stuart-Fox. 2012. Accelerated speciation in color polymorphic birds. *Letters to Nature,* **485**: 631-635. doi:10.1038/nature11050

Huxley J.S. 1955. Morphism and evolution. *Heredity*, **9**: 1–51. doi: 10.1038/hdy.1955.1

Jain S.K. 1976. The evolution of inbreeding in plants. *Annu. Rev. Ecol.Syst.*, **7**: 469–495. doi: 10.1146/annurev.es.07.110176.002345

Jiménez-López F.J., M. Talavera, P.L. Ortiz and M. Arista. 2015. Nuclear microsatellite primers in the annual herb *Lysimachia arvensis* (Myrsinaceae) and closely related taxa. *Biochem. Syst. Ecol.*, **58**: 242-246.

Jiménez-López F.J., M. Arista, M. Talavera, J.R. Pannell and P.L. Ortiz. 2019.Heritabilities of lateral and vertical herkogamy in Lysimachia arvensis. *Plant Spec. Biol*., **34**: 31-37. doi: 10.1111/1442-1984.12229

Jones K.N. and J.S. Reithel. 2001. Pollinator-mediated selection on a flower color polymorphism in experimental populations of *Antirrhinum* (Scrophulariaceae). *Am. J. Bot.*, **88**: 447-454.

Kagawa K. and G. Takimoto. 2016. Inaccurate color discrimination by pollinators promotes evolution of discrete color polymorphism in food-deceptive flowers. *Am. Nat.*, **187**: 194–204. doi: 10.5061/dryad.q4c60

Kalisz S., D.W. Vogler and K.M. Hanley. 2004. Context-dependent autonomous self-fertilization yields reproductive assurance and mixed mating. *Nature*, 430: 884.

Lande R. and D.W. Schemske. 1985. The evolution of self-fertilization and inbreeding depression in plants. 1. Genetics models. *Evolution*, **39**, 24–40. doi: 10.1111/j.1558-5646.1985.tb04077.x

Levin D.A. and E.T. Brack. 1995. Natural selection against white petals in Phlox. Evolution, **49**: 1017-1022.

Loveless M.D. and J.L. Hamrick. 1984. Ecological determinants of genetic structure in plant populations. *Annu. Rev. Ecol. Syst.*, **15**: 65–95. doi: 10.1146/annurev.es.15.110184.000433

Lowry D., J. Modliszewski, K. Wright, C. Wu and J. Willis. 2008. The strength and genetic basis of reproductive isolating barriers in flowering plants. *Philos. Trans. R. Soc. Lond. B*, **363**: 3009–3021. doi: 10.1098/rstb.2008.0064

Mable B.K., A.V.R. Dart, C.D. Berardo and L. Witham. 2005. Breakdown of self-incompatibility in the perennial *Arabidopsis lyrata* (Brassicaceae) and its genetic consequences. *Evolution*, **59**: 1437-1448.

Manzano-Piedras E., A. Marcer, C. Alonso-Blanco andF.X. Picó. 2014. Deciphering the adjustment between environment and life history in annuals: lessons from a geographically-explicit approach in *Arabidopsis thaliana*. *PloS one*, *9*:e87836.

Martin N.H. and J.H. Willis. 2007. Ecological divergence associated with mating system causes nearly complete reproductive isolation between sympatric *Mimulus* species. *Evolution*, **61**: 68–82. doi: 10.1111/j.1558-5646.2007.00006.x

Meirmans P.G. and P.H. Van Tienderen. 2004. GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms. Mol. Ecol. Notes, **4**: 792-794.

Meléndez-Ackerman E., D.R. Campbell and N.M. Waser. 1997. Hummingbird behavior and mechanisms of selection on flower color in *Ipomopsis*. *Ecology*, **78**: 2532-2541.

Michalski S.G. and W. Durka. 2007. High selfing and high inbreeding depression in peripheral populations of *Juncus atratus*. *Mol. Ecol.*, **16**: 4715-4727.

Morgan M.T. and D.J. Schoen. 1997. Selection on reproductive characters: floral morphology in *Asclepias syriaca*. *Heredity*, **79**: 433.

Narbona E., H. Wang, P.L. Ortiz, M. Arista, and E. Imbert. 2018. Flower colour polymorphism in the Mediterranean Basin: occurrence, maintenance and implications for speciation. *Plant Biol.*, **20**: 8–20. doi: 10.1111/plb.12575

Nosil P., L.J. Harmon, and O. Seehausen. 2009. Ecological explanations for (incomplete) speciation. *Trends Ecol. Evol.*, **24**: 145-156.

Ollerton J. and A. Lack. 1998. Relationships between flowering phenology, plant size and reproductive success in *Lotus corniculatus* (Fabaceae). *Plant Ecol.*, **139**: 35–47. doi: 10.1023/A:1009798320049

Ortiz P.L., R. Berjano, M. Talavera, L. Rodríguez-Zayas and M. Arista. 2015. Flower colour polymorphism in *Lysimachia arvensis*: how is the red morph maintained in Mediterranean environments? *Perspect. Plant Ecol. Evol. Syst.*, **17**: 142– 150. doi: 10.1016/j.ppees.2015.01.004

Pujadas A. 1997. *Anagallis* L. In Castroviejo S. et al. (Eds.) *Flora Iberica*. Vol. V. Real Jardín Botánico, CSIC. Madrid, Spain.

Rausher M.D. 2008. Evolutionary transitions in floral color. *Int. J. Plant Sci.*, **169**: 7– 21. doi: 10.1086/523358

Rausher M.D., D. Augustine, and A. VanderKooi. 1993. Absence of pollen discounting in a genotype of *Ipomoea purpurea* exhibiting increased selfing. *Evolution*, **47**: 1688-1695.

Raymond M. and F. Rousset. 2011. GENEPOP version 4.0.10. Available at [http://genepop.curtin.edu.au/]. Accessed November 27, 2017.

Rodríguez-Pérez J. and A. Traveset. 2016. Effects of flowering phenology and synchrony on the reproductive success of a long-flowering shrub. *AoB Plants*, *8*: plw007. doi: 10.1093/aobpla/plw007

Schenk J.H. and R.B. Jackson. 2002. The global biogeography of roots. *Ecol. Monogr.*, **72**: 311–328. doi: 10.1890/0012-9615(2002)072[0311:TGBOR]2.0.CO;2

Schiestl F.P. and S.D. Johnson. 2013. Pollinator-mediated evolution of floral signals. Trends Ecol. Evol., **28**: 307-315.

Schneider S., D. Roessli, and L. Excoffier. 2000. Arlequin: a software for population genetics data analysis. User manual v. 2.000. Genetics and Biometry Lab, Dept. of Anthropology, University of Geneva, Switzerland.

Schoen D.J. and A.H.D. Brown. 1991. Whole- and part-flower self-pollination in *Glycine clandestina* and *G. argyrea* and the evolution of autogamy. *Evolution* **45**: 1665–1674.

Servedio M.R., G.S. Van Doorn, M. Kopp, A.M. Frame and P. Nosil. 2011. Magic traits in speciation: 'magic'but not rare? *Trends Ecol. Evol.*, **26**: 389-397.

Shivanna K.R. 2015. Reproductive assurance through autogamous self-pollination across diverse sexual and breeding systems. *Curr. Sci.*, 109**:** 1255-1263.

Smithson A. and M.R. MacNair. 1996. Frequency-dependent selection by pollinators: mechanisms and consequences with regard to behaviour of bumblebees *Bombus terrestris* (L.) (Hymenoptera: Apidae). *J. Evol. Biol*. **9**: 571–588.

Sobral M., T. Veiga, P. Domínguez, J.A. Guitián, P. Guitián and J.M. Guitián. 2015. Selective pressures explain differences in flower color among *Gentiana lutea* populations. *PLoS One*, *10*: e0132522.

Strauss S.Y. and J.B. Whittall. 2006. Non-pollinator agents of selection on floral traits. Ecology and evolution of flowers (eds , Harder L.D. and S.C.H. Barrett), pp. 120–135. Oxford, UK: Oxford University Press.

Subramaniam B. and M.D. Rausher. 2000. Balancing selection on a floral polymorphism. *Evolution*, **54**: 691–695. doi: 10.1554/0014-3820(2000)054[0691:BSOAFP]2.0.CO;2

Takebayashi N. and P.P. Morrell. 2001. Is self-fertilization an evolutionary dead end? Revisiting an old hypothesis with genetic theories and a macroevolutionary approach. *Am. J. Bot*. **88**: 1143–1150.

Van Puyvelde K., A. Van Geert and L. Triest. 2010. ATETRA, a new software program to analyse tetraploid microsatellite data: comparison with TETRA and TETRASAT. *Mol. Ecol. Res*., **10**: 331-334. doi: 10.1111/j.1755-0998.2009.02748.x

Vos P., R. Hogers, M. Bleeker, M. Reijans, T.V.D. Lee, M. Hornes, A. Friters, J. Pot, J. Paleman, M. Kuiper and M. Zabeau. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic acids research*, **23**: 4407-4414. doi: 10.1093/nar/23.21.4407

Warnes G. and F. Leisch. 2005. Genetics: population genetics. *R package version*, **1**: 1-5.

Warren J. and S. Mackenzie. 2001. Why are all colour combinations not equally represented as flower-colour polymorphisms? *New Phytol.*, **151**: 237-241.

Waser N.M. and M.V. Price. 1981. Pollinator choice and stabilizing selection for flower color in *Delphinium nelsonii*. *Evolution*, **35**: 376-390.

Waser N.M. and M.V. Price. 1983. Pollinator behaviour and natural selection for flower colour in *Delphinium nelsonii*. *Nature*, **302**: 422.

Wessinger C.A. and M.D. Rausher. 2012. Lessons from flower colour evolution on targets of selection. *J. Exp. Bot.*, **63**: 5741–5749. doi: 10.1093/jxb/ers267

Whibley A.C., N.B. Langlade, C. Andalo, A.I. Hanna, A. Bangham, C. Thébaud and E. Coen. 2006. Evolutionary paths underlying flower color variation in *Antirrhinum*. *Science*, **313**: 963–966. doi: 10.1126/science.1129161

Whitney K.D. 2005. Linking frugivores to the dynamics of a fruit color polymorphism. *Am. J. Bot.*, **92**: 859–867. doi: 10.3732/ajb.92.5.859

Widmer A., C. Lexer and S. Cozzolino. 2009. Evolution of reproductive isolation in plants. *Heredity*, **102**: 31–38. doi: 10.1038/hdy.2008.69

Winn A.A., E. Elle, S. Kalisz, P.O. Cheptou, C.G. Eckert, C. Goodwillie, M.O. Johnston, D.A. Moeller, R.H. Ree, R.D. Sargent and M. Vallejo-Marín. 2011. Analysis of inbreeding depression in mixed-mating plants provides evidence for selective interference and stable mixed mating. *Evolution*, **65**: 3339-3359.

Table 6 SuppInfo: Individual and multi-locus gene diversity estimates for nine SSR microsatellites studied, for each population and colour morph of Lysimachia arvensis. Measurements were taken in six red-flowered and six blue-flowered plants in polymorphic populations and in ten plants in monomorphic populations. Population details are in Appendix 1, available online. Color (B, Blue; R, Red). Population type (P, polymorphic; M, monomorphic). A, Allele number per locus; Ho, observed heterozygosity; He, expected heterozygosity; Gis, inbreeding coefficient; No, null allele frecuency (**p<0.01; *p<0.05).

			Lys11					Lys 12					Lys16				
Pop	**Color**	**Type**	**A**	**Ho**	**He**	**Gis**	**No**	**A**	**Ho**	**He**	**Gis**	**No**	**A**	**Ho**	**He**	**Gis**	**No**
IT-Cer	B	P	2.703	0.700	0.663	-0.137	0.000	2.000	0.200	0.533	-0.167	0.380	2.215	0.200	0.577	-0.137	0.198
IT-Cer	R	P	1.853	0.400	0.485	0.115	0.028	2.631	0.300	0.653	0.015	0.135	1.000	0.000	0.000	---	---
TR	B	P	2.615	0.400	0.650	0.048	0.100	1.967	0.400	0.517	0.171	0.046	2.467	0.400	0.626	-0.056	0.000
TR	R	P	1.000	0.000	0.000	---	---	2.000	0.500	0.526	-0.267	0.000	1.000	0.000	0.000	---	---
ES-Av	B	P	3.298	0.300	0.733	-0.048	0.127	2.000	0.500	0.526	-0.267	0.000	2.466	0.300	0.626	-0.056	0.189
ES-Av	R	P	1.000	0.000	0.000	---	---	2.000	0.500	0.526	-0.065	0.000	1.000	0.000	0.000	---	---
PR-Az-1	B	P	1.000	0.000	0.000	---	---	2.215	0.400	0.577	-0.267*	0.000	1.000	0.000	0.000	---	---
PR-Az-1	R	P	2.000	0.250	0.526	-0.188	0.289	2.000	0.000	0.000	---	---	2.274	0.500	0.598	-0.292	0.000
IT-Sc	B	P	2.455	0.400	0.624	-0.047	0.083	2.050	0.225	0.546	-0.267	0.478	2.701	0.600	0.663	-0.095	0.000
IT-Sc	R	P	1.000	0.000	0.000	---	---	2.052	0.125	0.547	0.074	0.145	1.000	0.000	0.000	---	---
TN-1	B	P	2.849	0.600	0.683	-0.039	0.083	2.587	0.800	0.646	0.040	0.000	3.105	0.600	0.714	-0.000	0.070
TN-1	R	P	1.923	0.000	0.505	1.000**	0.327	3.000	0.625	0.704	-0.256**	0.000	1.000	0.000	0.000	---	---
GR-Cr	B	P	2.909	0.700	0.691	-0.197*	0.000	2.559	0.400	0.650	-0.081	0.315	2.748	0.800	0.670	-0.033	0.000
GR-Cr	R	P	1.471	0.000	0.337	1.000**	0.269	2.579	0.200	0.700	-0.098	0.287	1.000	0.000	0.000	---	---
MA-1	B	P	2.000	0.333	0.545	-0.019	0.000	2.508	0.500	0.656	0.193	0.158	3.000	0.600	0.727	-0.222	0.000
MA-1	R	P	1.000	0.000	0.000	---	---	2.629	0.318	0.634	-0.158*	0.102	1.000	0.000	0.000	---	---
ES-Ca-Zh	B	P	2.699	0.400	0.663	-0.184*	0.000	2.000	0.200	0.526	-0.188	0.000	3.473	0.800	0.750	-0.008	0.000
ES-Ca-Zh	R	P	1.000	0.000	0.000	---	---	2.909	0.400	0.700	-0.333**	0.000	1.000	0.000	0.000	---	---

			Lys11					Lys 12					Lys16				
Pop	**Color**	**Type**	**A**	**Ho**	**He**	**Gis**	**No**	**A**	**Ho**	**He**	**Gis**	**No**	**A**	**Ho**	**He**	**Gis**	**No**
TN-2	B	M	2.830	0.533	0.663	0.052	0.036	2.057	0.500	0.527	0.051	0.086	3.449	0.750	0.728	-0.055	0.000
ES-Co1	B	M	2.449	0.350	0.607	0.108	0.093	2.318	0.400	0.583	0.154*	0.167	3.355	0.650	0.720	-0.098	0.000
MA-2	B	M	2.773	0.444	0.657	-0.177*	0.000	1.767	0.400	0.445	-0.219*	0.000	3.033	0.750	0.687	-0.076	0.018
ES-Ma	B	M	2.848	0.600	0.665	-0.057	0.000	2.532	0.400	0.622	0.133	0.249	3.063	0.700	0.691	-0.047	0.036
ES-Co2	R	M	1.862	0.000	0.475	0.621**	0.335	1.185	0.200	0.160	0.235	0.055	3.000	0.500	0.684	-0.300**	0.000
ES-Ca-Gr	B	P	2.849	0.500	0.683	-0.080	0.000	2.000	0.000	0.000	-0.262*	---	3.071	0.600	0.710	-0.027	0.070
ES-Ca-Gr	R	P	1.000	0.000	0.000	---	---	3.101	0.550	0.720	---	0.000	1.000	0.000	0.000	---	---
ES-Te	B	P	3.030	0.200	0.705	0.338*	0.236	2.000	0.200	0.526	-0.188	0.000	1.000	0.000	0.000	---	---
ES-Te	R	P	3.607	0.400	0.761	0.035	0.161	2.615	0.625	0.652	0.009	0.076	2.273	0.500	0.597	-0.292	0.000
ES-Po	R	M	1.471	0.000	0.328	1.000**	0.269	2.086	0.100	0.534	0.761**	0.303	3.000	0.500	0.684	-0.300**	0.000
PR-Az-2	R	M	2.745	0.550	0.652	-0.090	0.000	2.179	0.500	0.555	0.202*	0.164	2.000	0.000	0.000	---	---
GR	R	M	2.000	0.000	0.513	1.000**	0.333	2.431	0.413	0.605	-0.094	0.082	2.666	0.500	0.643	-0.275**	0.000
CH	R	M	2.695	0.500	0.645	-0.039	0.032	2.909	0.563	0.674	-0.247	0.000	3.000	0.500	0.684	-0.300**	0.000
Overall			1.808	0.467	0.790	0.114**	0.117	2.208	0.575	0.668	-0.050**	0.111	1.697	0.430	0.817	-0.133**	0.031

			Lys28					Lys29					Lys30				
Pop	**Color**	**Type**	**A**	**Ho**	**He**	**Gis**	**No**	**A**	**Ho**	**He**	**Gis**	**No**	**A**	**Ho**	**He**	**Gis**	**No**
IT-Cer	B	P	2.719	0.633	0.669	-0.082	0.000	2.767	0.585	0.672	0.616**	0.246	5.200	0.500	0.862	0.314*	0.323
IT-Cer	R	P	2.041	0.100	0.537	0.394*	0.268	1.994	0.604	0.525	0.296*	0.330	1.991	0.100	0.531	0.521*	0.239
TR	B	P	1.364	0.200	0.284	0.375	0.398	1.674	0.780	0.424	0.725**	0.318	1.436	0.200	0.320	0.414*	0.196
TR	R	P	1.267	0.100	0.222	0.131	0.165	2.059	0.850	0.541	-0.092**	0.269	3.327	0.800	0.736	-0.089	0.000
ES-Av	B	P	2.000	0.100	0.526	-0.215	0.000	1.000	0.000	0.000	---	---	2.456	0.500	0.624	-0.191	0.000

			Lys28					Lys29					Lys30				
Pop	**Color**	**Type**	**A**	**Ho**	**He**	**Gis**	**No**	**A**	**Ho**	**He**	**Gis**	**No**	**A**	**Ho**	**He**	**Gis**	**No**
ES-Av	R	P	1.479	0.000	0.341	0.095	0.327	2.612	0.225	0.651	-0.111	0.100	2.701	0.350	0.665	-0.232*	0.000
PR-Az-1	B	P	2.701	0.567	0.667	-0.236*	0.000	1.671	0.095	0.423	0.725**	0.318	3.082	0.625	0.713	-0.140	0.000
PR-Az-1	R	P	2.030	0.200	0.534	-0.056	0.000	2.000	0.000	0.000	---	---	3.375	0.600	0.741	-0.101	0.000
IT-Sc	B	P	1.968	0.200	0.519	0.011	0.145	1.908	0.703	0.501	0.531**	0.330	2.314	0.300	0.598	0.440**	0.152
IT-Sc	R	P	3.000	0.500	0.722	-0.335*	0.000	2.000	0.000	0.000	---	---	1.844	0.125	0.488	0.727*	0.222
TN-1	B	P	2.248	0.225	0.586	0.244	0.198	1.267	0.400	0.222	0.208**	0.327	2.909	0.400	0.700	-0.143	0.000
TN-1	R	P	1.699	0.100	0.433	0.021	0.164	1.267	0.600	0.222	0.208**	0.327	2.676	0.200	0.663	-0.123	0.158
GR-Cr	B	P	2.849	0.300	0.687	-0.192	0.158	1.115	0.200	0.108	0.296**	0.269	1.892	0.100	0.519	0.074	0.241
GR-Cr	R	P	2.200	0.300	0.576	-0.091	0.016	2.290	0.100	0.595	-0.020**	0.194	2.778	0.000	0.674	1.000**	0.391
MA-1	B	P	3.000	0.500	0.727	-0.170	0.000	1.565	0.333	0.394	0.312	0.056	1.211	0.150	0.190	0.000	0.000
MA-1	R	P	2.509	0.384	0.616	-0.160*	0.073	2.285	0.182	0.576	-0.104	0.089	1.988	0.182	0.509	0.391**	0.217
ES-Ca-Zh	B	P	2.851	0.367	0.687	-0.214*	0.056	1.923	0.000	0.505	1.000**	0.327	2.635	0.600	0.653	0.102	0.000
ES-Ca-Zh	R	P	1.967	0.100	0.519	-0.080	0.198	1.267	0.200	0.222	0.406	0.083	4.010	0.700	0.790	-0.155*	0.000
TN-2	B	M	2.131	0.538	0.545	-0.227*	0.000	1.583	0.200	0.378	0.391*	0.110	2.332	0.600	0.586	-0.096	0.000
ES-Co1	B	M	2.104	0.200	0.538	-0.214	0.000	2.357	0.200	0.590	0.377**	0.210	2.978	0.150	0.681	0.636**	0.307
MA-2	B	M	2.454	0.283	0.609	-0.289**	0.000	2.180	0.106	0.556	0.684**	0.286	2.660	0.306	0.642	0.152	0.289
ES-Ma	B	M	3.125	0.525	0.698	-0.096	0.059	2.215	0.100	0.563	0.851**	0.283	3.483	0.200	0.732	0.490**	0.304
ES-Co2	R	M	1.000	0.000	0.000	---	---	2.941	0.400	0.677	-0.182**	0.000	1.471	0.000	0.328	1.000**	0.269
ES-Ca-Gr	B	P	2.701	0.500	0.663	-0.198*	0.000	1.923	0.000	0.505	1.000**	0.327	2.702	0.550	0.665	0.026	0.036
ES-Ca-Gr	R	P	2.701	0.567	0.667	-0.262*	0.000	1.964	0.000	0.517	0.548**	0.330	2.943	0.600	0.695	-0.031	0.000
ES-Te	B	P	1.850	0.367	0.486	-0.077	0.000	1.923	0.000	0.505	1.000**	0.251	2.604	0.200	0.650	0.344*	0.226
ES-Te	R	P	2.700	0.500	0.669	-0.251	0.000	2.215	0.100	0.579	-0.267**	0.000	2.055	0.425	0.542	0.050	0.000

			Lys28					Lys29					Lys30				
Pop	**Color**	**Type**	**A**	**Ho**	**He**	**Gis**	**No**	**A**	**Ho**	**He**	**Gis**	**No**	**A**	**Ho**	**He**	**Gis**	**No**
ES-Po	R	M	1.920	0.000	0.491	-0.182	0.204	3.448	0.600	0.728	-0.190**	0.000	3.390	0.100	0.723	0.908**	0.213
PR-Az-2	R	M	1.114	0.100	0.105	-0.026	0.000	2.234	0.263	0.568	-0.349**	0.156	2.024	0.400	0.519	0.136	0.102
GR	R	M	1.676	0.250	0.414	0.278*	0.102	3.398	0.106	0.724	0.126*	0.285	3.643	0.306	0.745	0.358**	0.248
CH	R	M	2.000	0.000	0.000	---	---	1.242	0.106	0.200	0.093	0.089	3.006	0.525	0.685	-0.088	0.042
Overall			1.997	0.523	0.654	-0.103**	0.087	1.839	0.477	0.657	0.263**	0.211	2.455	0.622	0.807	0.207**	0.135

			Lys31					Lys32					Lys33				
Pop	**Color**	**Type**	**A**	**Ho**	**He**	**Gis**	**No**	**A**	**Ho**	**He**	**Gis**	**No**	**A**	**Ho**	**He**	**Gis**	**No**
IT-Cer	B	P	3.198	0.900	0.723	-0.095	0.000	3.483	0.800	0.750	-0.063	0.000	2.069	0.400	0.544	0.187	0.061
IT-Cer	R	P	1.923	0.000	0.505	1.000**	0.333	1.000	0.000	0.000	---	---	2.533	0.100	0.637	0.073	0.135
TR	B	P	2.847	0.700	0.685	-0.081	0.028	3.032	0.300	0.705	0.372*	0.183	2.036	0.300	0.536	0.282*	0.134
TR	R	P	1.000	0.000	0.000	---	---	1.115	0.250	0.108	0.296	0.269	1.675	0.100	0.424	0.759**	0.234
ES-Av	B	P	2.000	0.300	0.526	-0.088	0.000	3.516	0.300	0.753	0.483	0.240	1.000	0.000	0.000	---	---
ES-Av	R	P	1.000	0.000	0.000	---	---	1.000	0.000	0.000	---	---	1.899	0.230	0.498	0.659**	0.251
PR-Az-1	B	P	2.232	0.200	0.581	0.563**	0.346	1.114	0.100	0.108	0.000	0.000	1.000	0.000	0.000	---	---
PR-Az-1	R	P	2.215	0.200	0.579	-0.221*	0.000	3.133	0.625	0.717	-0.087	0.000	1.000	0.000	0.000	---	---
IT-Sc	B	P	2.296	0.200	0.596	-0.023	0.226	3.509	0.500	0.753	0.083	0.103	1.626	0.225	0.407	0.050	0.145
IT-Sc	R	P	1.000	0.000	0.000	---	---	1.684	0.000	0.433	0.615**	0.291	2.729	0.854	0.676	0.091	0.290
TN-1	B	P	2.338	0.500	0.602	0.081	0.000	3.663	0.400	0.765	0.022	0.134	3.363	0.400	0.740	0.415	0.164
TN-1	R	P	1.000	0.000	0.000	---	---	1.117	0.100	0.111	-0.800*	0.224	2.632	0.225	0.655	-0.102**	0.1975
GR-Cr	B	P	2.905	0.650	0.692	0.003	0.000	3.458	0.700	0.748	0.009	0.036	1.408	0.100	0.305	0.174	0.232
GR-Cr	R	P	1.114	0.100	0.107	0.000	0.294	1.280	0.125	0.233	-0.714**	0.250	2.602	0.367	0.652	0.011	0.070

			Lys31					Lys32					Lys33				
Pop	**Color**	**Type**	**A**	**Ho**	**He**	**Gis**	**No**	**A**	**Ho**	**He**	**Gis**	**No**	**A**	**Ho**	**He**	**Gis**	**No**
MA-1	B	P	2.510	0.167	0.656	-0.014	0.245	3.491	0.333	0.778	0.065	0.176	1.000	0.000	0.000	---	---
MA-1	R	P	1.796	0.046	0.454	0.595**	0.301	1.246	0.091	0.203	0.161	0.320	1.000	0.000	0.000	---	---
ES-Ca-Zh	B	P	3.245	0.400	0.730	0.186	0.194	2.334	0.200	0.602	-0.038	0.229	1.841	0.300	0.481	0.008	0.269
ES-Ca-Zh	R	P	1.000	0.000	0.000	---	---	1.000	0.000	0.000	---	---	1.438	0.200	0.321	0.055	0.091
TN-2	B	M	2.827	0.350	0.663	0.006	0.076	3.272	0.650	0.712	0.094	0.034	1.789	0.150	0.453	0.365**	0.291
ES-Co1	B	M	3.612	0.500	0.742	0.104	0.102	3.044	0.450	0.691	-0.003	0.126	1.303	0.050	0.239	0.573**	0.191
MA-2	B	M	3.589	0.211	0.740	0.088	0.252	3.814	0.636	0.758	0.037	0.032	1.771	0.200	0.446	0.854**	0.319
ES-Ma	B	M	3.832	0.285	0.760	0.021	0.235	3.221	0.517	0.708	0.115	0.109	1.316	0.100	0.247	0.081	0.112
ES-Co2	R	M	1.000	0.000	0.000	---	---	1.515	0.000	0.349	1.000**	0.285	1.000	0.000	0.000	---	---
ES-Ca-Gr	B	P	2.536	0.200	0.638	0.040	0.269	3.750	0.500	0.772	0.022	0.086	1.000	0.000	0.000	---	---
ES-Ca-Gr	R	P	1.000	0.000	0.000	---	---	1.114	0.100	0.108	0.000	0.000	1.650	0.100	0.415	0.075	0.236
ES-Te	B	P	4.310	0.875	0.811	-0.025	0.066	3.557	0.550	0.759	0.091	0.075	1.000	0.000	0.000	---	---
ES-Te	R	P	3.241	0.200	0.728	0.026	0.241	4.050	0.200	0.797	-0.003	0.236	1.479	0.200	0.341	0.095	0.327
ES-Po	R	M	1.724	0.000	0.431	1.000**	0.337	2.381	0.000	0.595	1.000**	0.369	2.174	0.000	0.554	1.000**	0.357
PR-Az-2	R	M	1.947	0.161	0.499	0.188	0.285	3.629	0.578	0.744	-0.007	0.017	1.000	0.000	0.000	---	---
GR	R	M	1.520	0.150	0.351	0.190	0.286	1.000	0.000	0.000	---	---	2.707	0.300	0.647	0.705**	0.393
CH	R	M	1.768	0.350	0.445	0.064	0.316	2.454	0.450	0.608	-0.113	0.017	1.053	0.200	0.052	0.316*	0.204
Overall			1.784	0.460	0.794	0.126**	0.185	1.901	0.496	0.822	0.120	0.142	1.480	0.339	0.643	0.299**	0.214

Appendix 1. Population identity of samples included in molecular studies.

Populations	Localities	Longitude	Latitude	Voucher ID	Flower colour
IT-Cer	ITALY. Sardinia. Chia. Spiaggia Chia. Monte Cogoni	38°53'39.6"N	8°52'36.2"E	SEV252546	Blue
IT-Cer	ITALY. Sardinia. Chia. Spiaggia Chia. Monte Cogoni	38°53'39.6"N	8°52'36.2"E	SEV252547	Red
TR	TURKEY. Antalya. Pine forest near to hotel Belek	36°50'54.5"N	31°4'39.2"E	SEV252544-2	Blue
TR	TURKEY. Antalya. Pine forest near to hotel Belek	36°50'54.5"N	31°4'39.2"E	SEV252544-1	Red
ES-Av	SPAIN. Ávila. Poyales del Hoyo	40°10'35"N	5°09'27"W	SEV278771	Blue
ES-Av	SPAIN. Ávila. Poyales del Hoyo	40°10'35"N	5°09'27"W	SEV278773	Red
PR-Az-1	PORTUGAL. Azores. Faial island. Capelo	38°34'58.8"N	28°47'44.7"W	SEV275728	Blue
PR-Az-1	PORTUGAL. Azores. Faial island. Capelo	38°34'58.8"N	28°47'44.7"W	SEV275727	Red
IT-Sc	ITALY. Sicily. Between Scillato & Caltavuturo	37°50'34.4"N	13°54'14.3"E	SEV279204	Blue
IT-Sc	ITALY. Sicily. Between Scillato & Caltavuturo	37°50'34.4"N	13°54'14.3"E	SEV279201	Red
TN-1	TUNISIA. Tabarka. Road close to oued Kebir	36°57'4.4"N	8°46'9.6"E	SEV279162	Blue
TN-1	TUNISIA. Tabarka. Road close to oued Kebir	36°57'4.4"N	8°46'9.6"E	SEV279163	Red
GR-Cr	GREECE. CRETE. Aghia Pelagia	35°24'36"N	24°59'51"E	SEV279241	Blue
GR-Cr	GREECE. CRETE. Aghia Pelagia	35°24'36"N	24°59'51"E	SEV279245	Red
MA-1	MOROCCO. Tétouan. Aouchtame Bni Said.	35°29'36"N	5°8'39"W	SEV283613	Blue
MA-1	MOROCCO. Tétouan. Aouchtame Bni Said.	35°29'36"N	5°8'39"W	SEV283614	Red
ES-Ca-Zh	SPAIN. Cádiz. Zahara de los Atunes	36°06'23.9"N	5°49'34.3"W	SEV278757	Blue
ES-Ca-Zh	SPAIN. Cádiz. Zahara de los Atunes	36°06'23.9"N	5°49'34.3"W	SEV278759	Red
TN-2	TUNISIA. Aïndraham. Between Aïndrahan & Fernana	36°44'08.2"N	8°40'48.3"E	SEV279157	Blue
ES-Co1	SPAIN. Cordoba. Carcabuey	37°26'23.30"N	4º16'37"W	SEV279276	Blue
MA-2	MOROCCO. Tanger. Cap Spartel	35°45'56"N	5º56'3"W	SEV283630	Blue
ES-Ma	SPAIN. Balearic Islands. Formentera. Es Ca Mari	38°41'17"N	1º27'45"E	SEV252540	Blue

Populations	Localities	Longitude	Latitude	Voucher ID	Flower colour
ES-Co2	SPAIN. Cordoba. Carcabuey. Fuente Dura	37°27'03.8"N	4°16'44.3"W	SEV279258	Red
ES-Ca-Gr	SPAIN. Cádiz. Sierra de Grazalema. Puerto del Boyar	36°45'25.1"N	5°23'42.4"W	SEV279114-2	Blue
ES-Ca-Gr	SPAIN. Cádiz. Sierra de Grazalema. Puerto del Boyar	36°45'25.1"N	5°23'42.4"W	SEV279114-1	Red
ES-Te	SPAIN. Canary Islands. Tenerife. Teide	28º21'15"N	16º31'05"W	SEV287853	Blue
ES-Te	SPAIN. Canary Islands. Tenerife. Teide	28º21'15"N	16º31'05"W	SEV287852	Red
ES-Po	SPAIN. Pontevedra. Cies Islands	42°13'37"N	8°53'52"W	SEV248973	Red
PR-Az-2	PORTUGAL. Azores. Pico island. Misterio do Santa Luzia	38º33'24"N	28º26'28.8"W	SEV275723	Red
GR	GREECE. Etolia-Akarnania. Amfilochia	38º55'52.5"N	21º10'12.8"E	B100736822	Red
CH	SWITZERLAND. Därligen. Between Spiez & Interlaken	46º39'49"N	7º48'7"E	SEV279131	Red

5. Heritabilities of lateral and vertical herkogamy in *Lysimachia arvensis*.

Jiménez-López F.J., Arista M., Talavera M., J.R. Pannell & Ortiz P.L.

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Heritabilities of lateral and vertical herkogamy in *Lysimachia arvensis*

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ABSTRACT

Herkogamy, spatial separation between stigma and anthers within a flower, is important in regulating plant-mating system. We studied phenotypic variation and heritability of herkogamy traits in *Lysimachia arvensis* (=*Anagallis arvensis*) that shows both lateral and vertical herkogamy in the same flower, a rare strategy in flowering plants. Both lateral and vertical herkogamy showed continuous variation in 15 natural populations. Lateral herkogamy, measured as the angle between style and stamens, ranged from 5.6 to 66.5 degrees; vertical herkogamy ranged from reverse to approach herkogamy. Herkogamy traits were constant within plants but variable among plants and populations. Flowers with marked lateral herkogamy showed mainly reverse herkogamy, while flowers with low lateral herkogamy showed mainly approach herkogamy. Both herkogamy traits showed a high degree of narrow sense heritability (h^2=0.843 for lateral and h^2=0.635 for vertical herkogamy). We discuss the possibility that variation in both herkogamy traits among populations of *L. arvensis* is a consequence of differential selective pressures under different pollination environments.

Keywords: *Anagallis*, approach herkogamy, floral trait evolution, Primulaceae, reverse herkogamy

INTRODUCTION

Herkogamy is the spatial separation between stigma and anthers within a hermaphrodite flower (Webb & Lloyd, 1986) and appears in both self-incompatible and self-compatible species. In self-incompatible species, herkogamy reduces interference between sexual organs, e.g., preventing stigma clogging and improving pollen export (Webb & Lloyd, 1986). In self-compatible species, herkogamy may also play an important role in preventing self-fertilization (Webb & Lloyd, 1986). Diverse studies have shown that small differences in herkogamy result in different rates of self-pollen deposition (e.g. Ritland & Ritland, 1989; Robertson et al., 1994; Karron et al., 1997; Motten & Stone, 2000). Increased herkogamy can be selected in environments in which cross-pollination is favoured, e.g. to prevent selfing and inbreeding depression. In contrast, in environments in which pollinators or mates are limited, reduced herkogamy may be favoured by selection for reproductive assurance (e.g. Moeller & Geber, 2005; de Vos et al., 2012).

The precise modality of stigma-anther separation can take several forms, including vertical and lateral herkogamy. Vertical herkogamy involves a vertical displacement between the stigma and anthers and is by far the most common. Stigma may situate above anthers such that pollinators contact it before pollen, a situation termed approach herkogamy; alternatively, stigma may situate below anthers such that pollinators contact it after pollen, a situation termed reverse herkogamy (Webb & Lloyd, 1986). Approach herkogamy is the most frequent form of vertical herkogamy in self-incompatible species while approach and reverse herkogamy are equally frequent in self-compatible species (Lloyd & Webb, 1992; Opedal et al., 2017). Some plant species show stylar polymorphism, with individuals presenting either approach or reverse herkogamy; the genetic basis of such polymorphism has been studied in some species, revealing the action of one or two major loci (Barrett et al., 2000; Ushijima et al., 2012; Nowak et al., 2015). In other species, styles show continuous variation in height, with flowers ranging from reverse to approach herkogamy (Motten & Stone 2000; Takebayashi et al., 2006). Metric traits, as the length of style or the position of stamens, are usually controlled by several loci with small effects (Kulbaba & Worley, 2008), and covariation between floral organs is common (e.g., Conner & Sterling 1996; Herrera, 2001). Such continuous covariation could constrain the evolvability of herkogamy (Lande & Arnold, 1983). A much less common type of spatial separation between sexual organs is lateral herkogamy, in which the style is horizontally displaced

from the centre of the flower, forming an angle with the stamens. Lateral herkogamy has been described, e.g., in *Linum* (Ruiz-Martín et al., 2018) and *Centaurium* (Brys & Jacquemyn, 2011), but as far as we are aware its genetic architecture is unknown.

Lysimachia arvensis is a self-compatible species that, unusually, shows both vertical and lateral herkogamy. Flowers show lateral herkogamy during their first day of opening, but their styles subsequently move to a central position, showing vertical herkogamy on their second day (Jiménez-López et al., pers. obs.). The consecutive expression of two types of herkogamy in the same flower is uncommon and could represent a two-step barrier to self-pollination. *Lysimachia arvensis* is widely distributed, occurring both in stable habitats with a favourable pollination environment and in disturbed places where the pollination environment may be unfavourable and/or unpredictable. If herkogamy regulates the probability of self-pollen deposition, variation in herkogamy might thus reflect variation in outcrossing opportunities (e.g. Takebayashi et al., 2006; Herlihy & Eckert, 2007). On the other hand, variation in the expression of herkogamy might simply reflect developmental instability (Dongen, 2000; Debat & David, 2001) or low trait canalization (Waddington, 1942; Debat & David, 2001). Either way, knowledge of its genetic architecture, specifically its narrow-sense heritability, would be a useful first step towards understanding the potential for herkogamy to evolve in response to selection. Our study thus aimed to determine the heritability of both lateral and vertical components of herkogamy in *Lysimachia arvensis*, as well as to characterize other correlations between floral traits.

MATERIAL AND METHODS

Lysimachia arvensis (L.) U. Manns & Anderb. (former *Anagallis arvensis* L.; Manns & Anderberg, 2009) is an annual plant native to the Mediterranean Basin but widely distributed around the world. The species is polymorphic in flower colour, with some plants producing blue flowers and other red flowers (Arista et al., 2013). Flowers last two or three days. Anther opening occurs during the first anthesis day and the stigma is receptive throughout the life span of the flower. During 2015 and 2016, we sampled 15 natural populations of *L. arvensis* in a wide geographical range during the peak of flowering (Appendix 1). In each population, we randomly collected between ten and 30 plants and measured herkogamy traits for two flowers per plant. Moreover, we studied floral variation within maternal families growing in a common glasshouse. In one population from Huelva (SW Spain; 37°17′31″N 6°22′43″W), seeds were collected in 2016 from a single fruit of each of eight individuals that were separated in the

population by at least 5 m. Seeds were allowed to germinate in a growth chamber, and the resulting plant families were raised in a glasshouse. We chose a single parent plant per family and measured its two herkogamy traits. Each such parent was hand-pollinated, either with pollen from another parent, or with its own pollen. Fruits resulting from each female-male parent combination were separately collected, and seeds were sown to obtain offspring families. Herkogamy traits were measured in 2017 for 6 to 16 offspring per family.

Herkogamy measurements were taken from fresh flowers, starting their first day of anthesis, based on photographs taken directly in the glasshouse. From these images, the two components of herkogamy were measured using ImageJ software. Lateral herkogamy was measured as the angle between style and stamens (hereafter 'style-stamen angle'; Fig. 1). To characterize approach or reverse herkogamy, we measured stamen length (from flower base to anther centre) and pistil length (from flower base to stigma centre; Fig. 1); approach or reverse herkogamy was then calculated as the difference between pistil and stamen lengths, which would be equivalent to final separation between anthers and stigma (hereafter 'stigma-anther displacement').

Variation in herkogamy traits among populations and plants was tested by means of general linear models (GLM), with population treated as a main effect and plant nested in population. Gaussian distributions were used to analyse both lateral and vertical herkogamy, and analyses were conducted using GLzM module of SPSS (IBM SPSS Statistic 24, USA) with Type III test. Pearson correlations between pistil and stamen lengths, stigma-anther displacement and style-stamen angle were calculated for the flowers measured in the field. To determine whether herkogamy traits were constant within plants, we performed Pearson correlations between the herkogamy values obtained in the two flowers of each plant sampled in the field. Heritability of each herkogamy trait was estimated for the plants growing in glasshouse by regressing values of offspring families on mean values of each trait of their parents; the slope of the line of best-fit is an estimate of heritability (h^2; e.g. Lennartsson et al., 2000).

RESULTS

Flowers of *Lysimachia arvensis* display two types of herkogamy that change sequentially throughout their life span. The first day of opening, all the flowers showed lateral herkogamy and the anthers were longitudinally placed in relation to stamen filament opening towards the inner of the flower (Fig. 1). During the second day of opening, the style had moved upright, and the anthers were transversally placed in

relation to the stamen filament (Fig. 1). In flowers lasting three days, vertical herkogamy did not change from the second day of anthesis.

In plants measured in the field, mean pistil length was 2.53 mm and mean stamen length was 2.50 mm (n = 600). Style-stamen angle ranged from 5.6 to 66.5 degrees, with a mean of 28.6 (n = 600) while stigma-anther displacement ranged from negative to positive values (mean 0.03, n = 600; Fig. 2). These four traits were significantly correlated (Fig. 3). Style-stamen angle was negatively correlated with both pistil and stamen length, while stigma-anther displacement was positively correlated with both traits. The two herkogamy traits were almost identical within plants, with Pearson correlations for both traits between pairs of flowers of the same plant being very high: $r = 0.939$ ($p < 0.0001$, n = 300) for style-stamen angle; $r = 0.885$ ($p < 0.0001$, n = 300) for stigma-anther displacement. Style-stamen angle and stigma-anther displacement showed significant variation among populations (Wald-chi square = 429.19, 14 df, $p < 0.001$ for lateral and Wald-chi square = 509.42, 14 df, $p < 0.001$ for vertical herkogamy) and plants (Wald-chi square = 9529.88, 283 df, $p < 0.001$ for lateral and Wald-chi square = 4310.46, 283 df, $p < 0.001$ for vertical herkogamy).

For plants coming from a single population and grown in the glasshouse, pistil length ranged from 0.90 to 2.80 mm (mean = 2.46, SD = 0.21, n = 96) and stamen length between 2.11 – 2.72 mm (mean = 2.36, SD = 0.15, n = 96). Style-stamen angle ranged from 12.56 – 40.71 degrees, with a mean angle of 25.38 degrees (SD = 6.89, n = 96) while stigma-anther displacement ranged from -0.06 to 0.42 mm with a mean 0.1 mm (SD = 0.1, n = 96). Heritabilities calculated in terms of offspring-parent regressions were significant for pistil length ($F_{1, 95} = 306.42$, $p < 0.0001$), stamen length ($F_{1, 95} = 328.09$, $p < 0.0001$), style-stamen angle ($F_{1, 95} = 230.98$, $p < 0.0001$; Fig. 4) and stigma-anther displacement ($F_{1, 95} = 63.68$, $p < 0.0001$; Fig. 4). Narrow-sense heritability (h^2) was high for all the traits: 0.875 for pistil length, 0.882 for stamen length, 0.843 for lateral herkogamy and 0.635 for vertical herkogamy.

DISCUSSION

Lysimachia arvensis displays two types of herkogamy that show continuous variation. The first day of opening, all the flowers observed showed lateral herkogamy, although in some cases the angle between the style and the stamens was as little as 5.6 degrees. However, in the second day of opening, the style had moved upright, and flowers showed wide and continuous variation in vertical herkogamy, ranging from reverse to approach herkogamy, and including flowers with their stigma and anthers at

the same level. Both herkogamy traits showed a high variation among plants, as has been found in other species (e.g., Luijten et al., 1999; Lennartsson et al., 2000; Herlihy & Eckert, 2007). Although we do not yet know the role of approach and reverse herkogamy in self-pollination in *L. arvensis*, flowers without vertical herkogamy are certainly able to self-pollinate autonomously (Jiménez-López, unpub. data). Thus, during their first day of opening, flowers of *L. arvensis* are more likely to be outcrossed, but those that do not show vertical herkogamy may show delayed self-pollination. Such delayed selfing is likely to be adaptive in an annual plant by conferring reproductive assurance in the absence of mates or pollinators (Kalisz & Vogler, 2003; Kalisz et al., 2004).

The lengths of pistil and stamens in *L. arvensis* were strongly correlated, i.e., flowers with longer pistils also had longer stamens. Both sexual organs also showed a positive correlation with vertical herkogamy, although variation in the stigma-anther displacement depended mainly on variation in pistil length. As indicated by the coefficients of determination (r^2), the length of the pistil was responsible for 51.2% of variation in stigma-anther displacement (Fig. 3B), while the length of the stamen was responsible for only 2.9% (Fig. 3D). That is, vertical herkogamy is mainly a consequence of variation in pistil length, and this may suggest that the correlation between sexual organs does not constrain the evolution of this trait in response to selective pressures (Ushimaru & Nakata, 2002). A similar result has been found for other taxa with continuous variation in vertical herkogamy, such as *Mimulus* (Kleunen & Ritland, 2004), *Aquilegia* (Herlihy & Eckert, 2007) and *Polemonium* (Kulbaba & Worley, 2008), as well as in species with stylar polymorphisms (e.g., Barrett et al., 2000). In contrast with vertical herkogamy, sexual organs showed a negative correlation with lateral herkogamy. Although the style-stamen angle does not necessarily depend on the lengths of the sexual organs, in *L. arvensis* this does appear to be the case. This implies that flowers with marked lateral herkogamy show mainly reverse vertical herkogamy, whereas flowers with low lateral herkogamy show mainly approach vertical herkogamy, as shown by the negative correlation between both herkogamy traits. *Lysimachia arvensis* only offers pollen as a reward, and bees grab the anthers to collect pollen, landing either directly on the anthers or on the petals and going quickly to the anthers. Thus, in flowers of *L. arvensis* with lateral herkogamy pollinators would contact the anthers before the stigma in most visits, which makes lateral herkogamy functionally similar to reverse vertical herkogamy. It would be interesting to determine the relative role of each herkogamy trait in preventing self-pollination, and whether they act synergistically.

Herkogamy traits were very similar within plants, as suggested by the strong correlations between the two measured flowers of each plant. This indicates that variation in herkogamy traits is unlikely to be a consequence of developmental instability (Dongen, 2000; Debat & David, 2001) and suggests strong broad-sense heritability. In fact, regressions of progeny on parent traits showed a high degree of narrow-sense heritability for both lateral and vertical herkogamy. Heritability of vertical herkogamy has been shown in numerous species, ranging from 0.30 to 0.85 (e.g., Luijten et al., 1999; Lennartsson et al., 2000; Kleunen & Ritland, 2004; Kulbaba & Worley, 2008): our estimate (h^2=0.635) is towards the high end of variation documented among species studied to date. Heritability of the lateral herkogamy (h^2=0.843) was even higher than that of vertical herkogamy. Heritability estimates for lateral herkogamy are much less frequent in the literature and, to our knowledge, our estimate seems to be the first.

In summary, our results indicate that both herkogamy traits in *L. arvensis* are phenotypically variable and have a high degree of heritability. If we assume that herkogamy affects self-pollen deposition as happens in other studied species (e.g. Barrett & Shore, 1987; Kalisz & Vogler, 2003; Kalisz et al., 2004; Takebayashi et al., 2006), differences in pollination environment can select for different herkogamy traits.

Lysimachia arvensis is a widely distributed species that occurs mainly in ruderal environments where pollinator availability is very variable. In fact, marked differences in pollinator attendance has been previously reported, with some populations of Southern Spain receiving very few visits (Gibbs & Talavera, 2001), others being visited by solitary bees (Ortiz et al., 2015) or other populations from Germany receiving visits from *Bombus terrestris* (Raine & Chittka, 2007). Thus, the variation in herkogamy traits found among populations could be a consequence of differential selective pressures by pollinators. Differences in pollinator attendance between colour morphs have also been reported, with the blue flowered morph receiving more visits than the red flowered morph (Ortiz et al., 2015). Although we have not measured differences in herkogamy traits between colour morphs, it is tempting to speculate a lower herkogamy expression in the less visited morph to increase reproductive assurance. Either way, knowing how each herkogamy trait prevents self-pollen deposition in *L. arvensis* would be crucial to understand their evolution.

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REFERENCES

Arista M., Talavera M., Berjano R. & Ortiz P. L. (2013) Abiotic factors may explain the geographical distribution of flower colour morphs and the maintenance of colour polymorphism in the scarlet pimpernel. *Journal of Ecology* **101**: 1613–1622.

Barrett S. C., Jesson L. K. & Baker A. M. (2000) The evolution and function of stylar polymorphisms in flowering plants. *Annals of Botany* **85**: 253–265.

Barrett S. C. H. & Shore J. S. (1987) Variation and evolution of breeding systems in the *Turnera ulmifolia* L. complex (Turneraceae). *Evolution* **41:** 340-354.

Brys R. & Jacquemyn H. (2011) Variation in the functioning of autonomous self-pollination, pollinator services and floral traits in three *Centaurium* species. *Annals of Botany* **107**: 917–925.

Conner J. K. & Sterling A. (1996) Selection for independence of floral and vegetative traits: evidence from correlation patterns in five species. *Canadian Journal of Botany* **74**: 642–644.

de Vos J. M., Keller B., Isham S. T., Kelso S. & Conti E. (2012) Reproductive implications of herkogamy in homostylous primroses: variation during anthesis and reproductive assurance in alpine environments. *Functional Ecology* **26**: 854–865.

Debat V. & David P. (2001) Mapping phenotypes: canalization, plasticity and developmental stability. *Trends in Ecology & Evolution* **16**: 555-561.

Dongen V. (2000) The evolutionary potential of developmental instability. *Journal of Evolutionary Biology*, **13**: 326-335.

Gibbs P. E. & Talavera S. (2001) Breeding system studies with three species of *Anagallis* (Primulaceae): self-incompatibility and reduced female fertility in *A. monelli* L. *Annals of Botany* **88**: 139-144.

Herlihy C. R. & Eckert C. G. (2007) Evolutionary analysis of a key floral trait in *Aquilegia canadensis* (Ranunculaceae): genetic variation in herkogamy and its effect on the mating system. *Evolution* **61**: 1661–1674.

Herrera J. (2001) The variability of organs differentially involved in pollination, and correlations of traits in Genisteae (Leguminosae: Papilionoideae). *Annals of Botany* **88**: 1027–1037.

Kalisz S. & Vogler D. W. (2003) Benefits of autonomous selfing under unpredictable pollinator environments. *Ecology* **84:** 2928-2942.

Kalisz S., Vogler D.W. & Hanley K. M. (2004) Context-dependent autonomous self-fertilization yields reproductive assurance and mixed mating. *Nature* **430:** 884–887.

Karron J. D., Jackson R. T., Thumser N. N. & Schlicht S. L. (1997) Outcrossing rates of individual *Mimulus ringens* genets are correlated with anther–stigma separation. *Heredity* **79**: 365–370.

Kleunen M. V. & Ritland K. (2004) Predicting evolution of floral traits associated with mating system in a natural plant population. *Journal of Evolutionary Biology* **17**: 1389–1399.

Kulbaba M. W. & Worley A. C. (2008) Floral design in *Polemonium brandegei* (Polemoniaceae): genetic and phenotypic variation under hawkmoth and hummingbird pollination. *International Journal of Plant Sciences* **169**: 509–522.

Lande R. & Arnold S. J. (1983) The measurement of selection on correlated characters. *Evolution* **37**: 1210-1226.

Lennartsson T., Oostermeijer J. G. B., van Dijk J. & den Nijs H. C. (2000) Ecological significance and heritability of floral reproductive traits in *Gentianella campestris* (Gentianaceae). *Basic and Applied Ecology* **1**: 69–81.

Lloyd D. G. & Webb C. J. (1992) The Evolution of Heterostyly. In: Barrett S. C. H. (ed). *Evolution and Function of Heterostyly.* Monographs on Theoretical and Applied Genetics, vol 15. Springer, Berlin, pp. 151-178.

Luijten S. H., Oostermeijer J. G. B., Ellis-Adam A. C. & den Nijs J. H. C. (1999) Variable herkogamy and autofertility in marginal populations of *Gentianella germanica* in the Netherlands. *Folia Geobotanica* **34**: 483.

Manns U. & Anderberg A. A. (2009) New combinations and names in Lysimachia (Myrsinaceae) for species of Anagallis, Pelletiera and Trientalis. *Willdenowia 39*: 49-54.

Moeller D. A. & Geber M. A. (2005) Ecological context of the evolution of self-pollination in *Clarkia xantiana*: population size, plant communities, and reproductive assurance. *Evolution* **59**: 786–799.

Motten A. F. & Stone J. L. (2000) Heritability of stigma position and the effect of stigma-anther separation on outcrossing in a predominantly self-fertilizing weed, *Datura stramonium* (Solanaceae). *American Journal of Botany* **87***:* 339–347.

Nowak M. D., Russo G., Schlapbach R., Hu C. N., Lenhard M. & Conti E. (2015) The draft genome of *Primula veris* yields insights into the molecular basis of heterostyly. *Genome biology* **16**: 12.

Opedal Ø. H., Bolstad G. H., Hansen T. F., Armbruster W. S. & Pélabon C. (2017) The evolvability of herkogamy: Quantifying the evolutionary potential of a composite trait. *Evolution* **71**: 1572–1586.

Ortiz P. L., Berjano R., Talavera M., Rodríguez-Zayas L. & Arista M. (2015) Flower colour polymorphism in *Lysimachia arvensis*: How is the red morph maintained in Mediterranean environments? *Perspectives in Plant Ecology, Evolution and Systematics*, **17**: 142-150.

Raine N.E. & Chittka L. (2007) The adaptive significance of sensory bias in a foraging context: floral colour preferences in the bumblebee *Bombus terrestris*. *PLoS One*, 2, e556.

Ritland C. & Ritland K. (1989) Variation of sex allocation among eight taxa of the *Mimulus guttatus* species complex (Scrophulariaceae). *American Journal of Botany* **67**: 1731–1739.

Robertson A. W., Diaz A. & Macnair M. R. (1994) The quantitative genetics of floral characters in *Mimulus guttatus*. *Heredity* **72**: 300–311.

Ruiz-Martín J., Santos-Gally R., Escudero M., Midgley J. J., Pérez-Barrales R. & Arroyo J. (2018) Style polymorphism in *Linum* (Linaceae): a case of Mediterranean parallel evolution? *Plant Biology* **20**: 100–111.

Takebayashi N., Wolf D. E. & Delph L. F. (2006) Effect of variation in herkogamy on outcrossing within a population of *Gilia achilleifolia*. *Heredity* **96**: 159-165.

Ushijima K., Nakano R., Bando M., Shigezane Y., Ikeda K., Namba Y., Kume S., Kitabata T., Mori H. & Kubo Y. (2012) Isolation of the floral morph-related genes in heterostylous flax (*Linum grandiflorum*): the genetic polymorphism and the transcriptional and post-transcriptional regulations of the S locus. *The Plant Journal* **69**: 317-331.

Ushimaru A. & Nakata K. (2002) The evolution of flower allometry in selfing species. *Evolutionary Ecology Research*, **4**: 1217-1227.

Waddington C. H. (1942) Canalization of development and the inheritance of acquired characters. *Nature* **150**: 563.

Webb C. J. & Lloyd D. G. (1986) The avoidance of interference between the presentation of pollen and stigmas in angiosperms II. Herkogamy. *New Zealand Journal of Botany* **24:** 163-178.

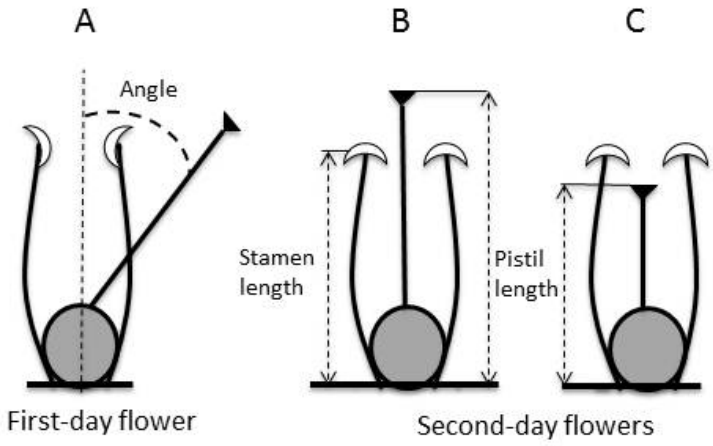

Figure 1. Schematic representation of flowers of *Lysimachia arvensis* on the first and the second day of anthesis, showing the traits measured to characterize herkogamy. A, lateral herkogamy; B, approach herkogamy; C, reverse herkogamy.

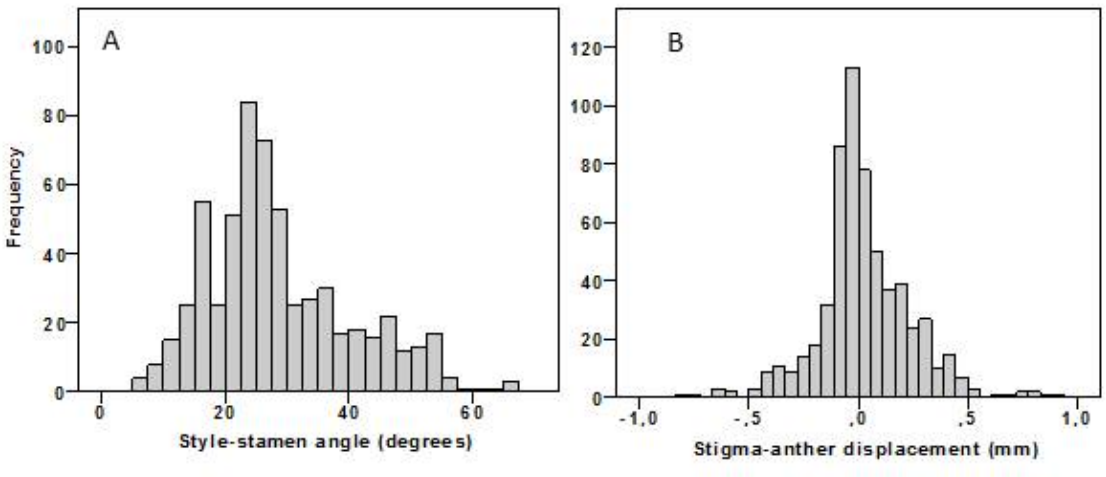

Figure 2. (A) Frequency distribution of style-stamen angle (degrees) in the first day of flower opening and (B) the stigma-anther displacement in the second day of flower opening in *Lysimachia arvensis* from 15 natural populations.

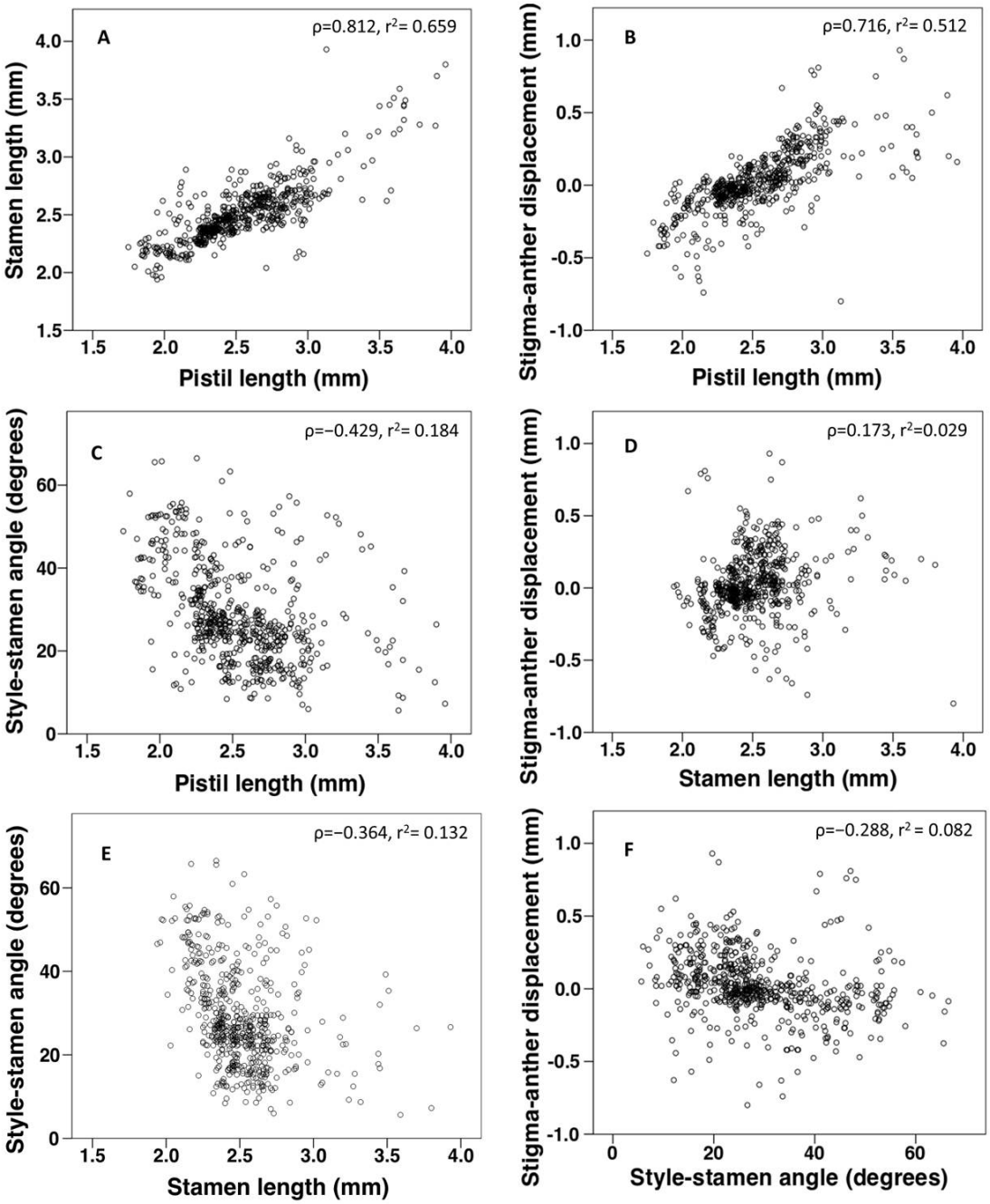

Figure 3. Pearson correlations between floral traits of 15 populations of *Lysimachia arvensis* in the W Europe and N Africa. (A) Pistil/Stamen length, (B) Pistil length/Stigma-anther displacement, (C) Pistil length/Style-stamen angle, (D) Stamen length/Stigma-anther displacement (E) Stamen length/Style-stamen angle and (F) Style-stamen angle/stigma-anther displacement. Sample sizes are 600 in all cases. All correlations were significant at $p < 0.0001$.

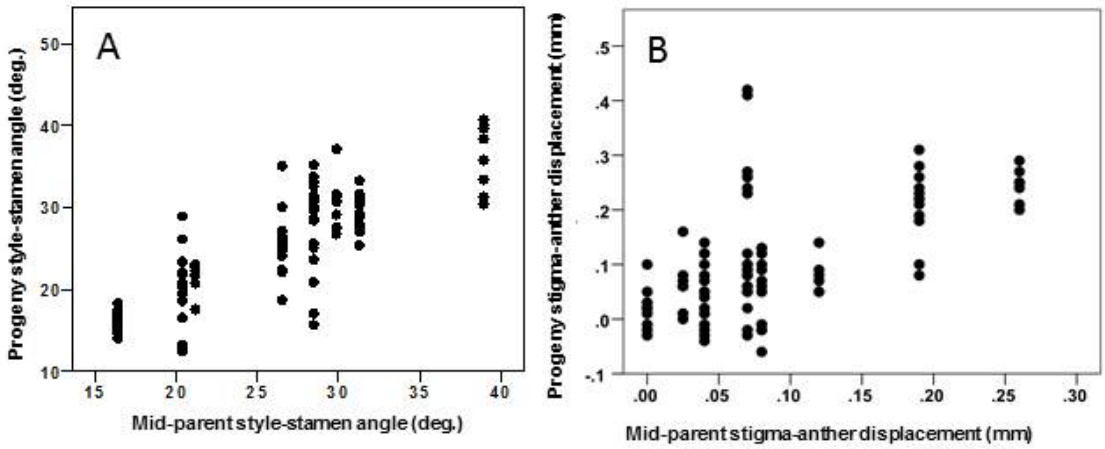

Figure 4. Relationships between the style-stamen angle (A) and the anther-stigma displacement (B) of parent plants of *Lysimachia arvensis* and their offspring derived from hand pollinations. Herkogamy values of parent plants were calculated as the mean value between both parents (i.e., mid-parent value).

6. Variation in lateral and vertical herkogamy between floral colour lineages of *Lysimachia arvensis* in allopatric and sympatric populations and its effects on mating systems.

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(Jiménez-López F.J., Ortiz P.L., Talavera M., Pannell J.R. & Arista M. Variation in lateral and vertical herkogamy between floral colour lineages of *Lysimachia arvensis* in allopatric and sympatric populations and its effects on mating systems. Enviando para su publicación en *New phytologist,* primera revisión)

ABSTRACT

(1) Herkogamy modulates the rate of self- versus outcross pollination and may regulate the extent of cross-lineage hybridisation when divergent evolutionary lineages co-occur. Although substantial evidence that herkogamy limits selfing exists, much less is known about the efficacy of lateral versus vertical herkogamy.

(2) We studied 25 populations of *Lysimachia arvensis,* a species in which two lineages with different flower colours co-occur. *Lysimachia arvensis* flowers display an unusual pattern of herkogamy, with lateral herkogamy first and vertical herkogamy subsequently. We characterised herkogamy of both lineages in allopatry and sympatry, and related herkogamy and autonomous selfing. Because hybrids between lineages have reduced fitness, we predicted greater selfing capacity in sympatry.

(3) Lateral herkogamy was more effective than vertical herkogamy in limiting self-pollen deposition, and in vertical herkogamy only approach herkogamy was effective. Lineages showed consistent differences in both herkogamy traits. Values of both herkogamy traits were lower in sympatry, but a greater self-pollen deposition was only observed for the predominant blue lineage.

(4) Our results contribute to understand how not only the degree but also the direction of herkogamy affects plant mating, as well as the evolution of reduced herkogamy as a mechanism to avoid reproductive interference between divergent lineages.

Key words: *Anagallis*, colour polymorphism, herkogamy, mating system, reproductive assurance, reproductive isolation, selfing evolution, self-pollen deposition.

INTRODUCTION

Patterns of mating in self-compatible plant populations depend to a large extent on the relative spatial conformation of male and female organs within and among individuals. This applies both to spatial separation of male (staminate) and female (pistillate) flowers in monoecious and dioecious species (known as 'dicliny'), but particularly to that between the anthers and stigmas of bisexual flowers ('herkogamy'). Herkogamy limits self-pollination (Brunet & Eckert, 1998; Motten & Stone, 2000; Takebayasi et al., 2006; reviewed in Opedal, 2018), and reduced herkogamy may allow plants autonomous self-pollination, with the potential advantage of reproductive assurance in situations where pollinators are rare or absent (Toräng et al., 2017). Indeed, the rate of self-fertilization is often high in self-compatible populations in which anthers are positioned close to stigmas, and the exclusion of pollinators has been shown to bring about the rapid evolution of reduced stigma-anther separation in experimental populations (Roels & Kelly, 2011; Brys & Jacquemin, 2012). Selection on the mating system may thus act directly through aspects of floral morphology, such as herkogamy, that regulate the capacity for self-pollen deposition or its prevention (Toräng et al., 2017). In situations in which divergent populations or potentially hybridizing species co-occur, reduced herkogamy may prevent gene flow between them, thus avoiding potentially deleterious hybridization and resulting in reproductive character displacement (Pfennig & Pfennig, 2009). For example, an increased capacity for selfing and reproductive divergence between hybridizing populations has been previously reported in closely co-occurring species of Centaurium (Brys et al., 2014), Mimulus (Martin & Willis, 2007; Grossenbacher & Whittall, 2011), and Clerodendrum (Miyake & Inoue, 2003).

Herkogamy may also influence mating among individuals with divergent floral strategies. This is particularly striking in heterostylous species, in which anthers are displaced vertically from one another ('vertical herkogamy'), held either above ('approach herkogamy') or below stigmas ('reverse herkogamy'), or in enantiostylous species, in which anthers are displaced laterally from stigmas to the right or left ('lateral herkogamy'). Both heterostyly and enantiostyly promote disassortative mating, such that pollen is transferred less frequently among individuals with the same conformation of floral parts than between those with different morphology (Lloyd & Webb, 1992; Barrett et al., 2000; 2006). Most species with vertical herkogamy comprise only one of the two conformations, with approach herkogamy more frequent than reverse (Webb & Lloyd, 1986; Barrett, 2003). Although more unusual, some species show continuous variation in the relative vertical positions of stigmas and anthers, ranging from reverse to approach herkogamy, e.g., *Datura stramonium* (Motten & Stone 2000) and *Gilia*

achilleifolia (Takebayashi et al., 2006). By contrast, just as enantiostyly is less common that heterostyly, so monomorphic lateral herkogamy is also relatively rare, e.g., *Centaurium* species (Brys et al., 2014, 2016).

Much has been learned about effects of vertical and lateral herkogamy on mating through the study of heterostylous (Lloyd & Webb, 1992) and enantiostylous (Jesson & Barrett, 2005) species, as well as in species with other stylar polymorphisms such as 'flexistyly' (Barrett, 2002), but their relative effectiveness in reducing self-pollen deposition or promoting outcrossing is not well known. Approach herkogamy tends to be better at reducing self-pollen deposition than reverse herkogamy (Motten & Stone 2000; Barrett, 2003; Takebayashi et al., 2006), although in general data on self-pollen deposition under reverse herkogamy are not common (Motten & Stone 2000; Takebayashi et al., 2006). The effectiveness of lateral herkogamy has been rarely reported, but in the few species studied self-pollination is reduced when the degree of herkogamy is high (Brys & Jacquemyn, 2011, 2012).

Importantly, the degree of herkogamy can change over the course of a flower's life, altering not only the amount of self-pollen deposition over time, but also the mode of self-fertilization and thus the relative costs and benefits of selfing. Three types of autonomous selfing have been recognised: 'prior selfing' (selfing before opportunities for outcrossing); 'competing selfing' (selfing at the same time as outcrossing, such that self and outcross pollen compete directly); and 'delayed selfing' (self-pollination after opportunities for outcrossing have been exhausted) (Lloyd, 1979). These types incur different costs that depend on cross pollen availability and the impact of inbreeding depression (Lloyd, 1979; Harder & Routley, 2006). Under prior and competing selfing, the potential for outcrossed success is reduced, and plants incur a cost of pollen and/or ovule discounting (Holsinger et al., 1984; Lloyd, 1992). In contrast, since delayed selfing occurs after outcrossing has been possible, the costs of pollen or ovule discounting are lower (Lloyd 1979, 1992; Herlihy & Eckert 2002; Harder & Routley, 2006). Delayed selfing should be particularly favoured in species with only a single chance to reproduce, such as annuals (Shivanna, 2015). Given the important role of herkogamy in general in regulating within-individual, between-individual and between-species mating, we ought to expect the temporal dynamics of herkogamy (i.e., anther and or stigma movement) during the course of flowering to respond to selection as a function of variation in outcrossing opportunities, the risk of inbreeding, or the risk of deleterious hybridization between genetically divergent lineages.

Within- and among-population variation in herkogamy presented by the annual self-compatible forb *Lysimachia arvensis* (L.) U. Manns & Anderb. (Primulaceae) offers an outstanding opportunity to assess the functional implications of vertical versus lateral

herkogamy in a context where easily identifiable genetically divergent lineages also co-occur. *Lysimachia arvensis* occurs around the Mediterranean Basin and has been introduced widely around the world. Unusually, it presents both vertical and lateral herkogamy (Jimenez-Lopez et al., 2019), and it comprises two flower-colour 'morphs' that appear to be partially reproductively isolated from one another (Jiménez-López et al., unpub. data). *L. arvensis* flowers show lateral herkogamy when they first open on day one, with anthers separated from the pistil as a result of an angular displacement of the stamens from the pistil, but the angle of separation then closes on the second and third days, so that anthers find themselves centrally on the same vertical axis as the pistil, either above, next to, or below the stigma (Jiménez-López et al., 2019; Fig. 1). The opportunity for self-pollen deposition in *L. arvensis* may thus not only vary among individuals that differ in the relative lengths of their pistils and/or stamens, but it may also vary over time (Jiménez-López et al., unpub. data). Both modes of herkogamy in *L. arvensis* have high heritability, with h^2 = 0.843 and 0.635 for lateral and vertical herkogamy, respectively (Jiménez-López et al., 2019). This variation allows a direct assessment, based on unmanipulated plants, of the distribution of herkogamy within and among populations, as well as of its implications for self-pollen deposition.

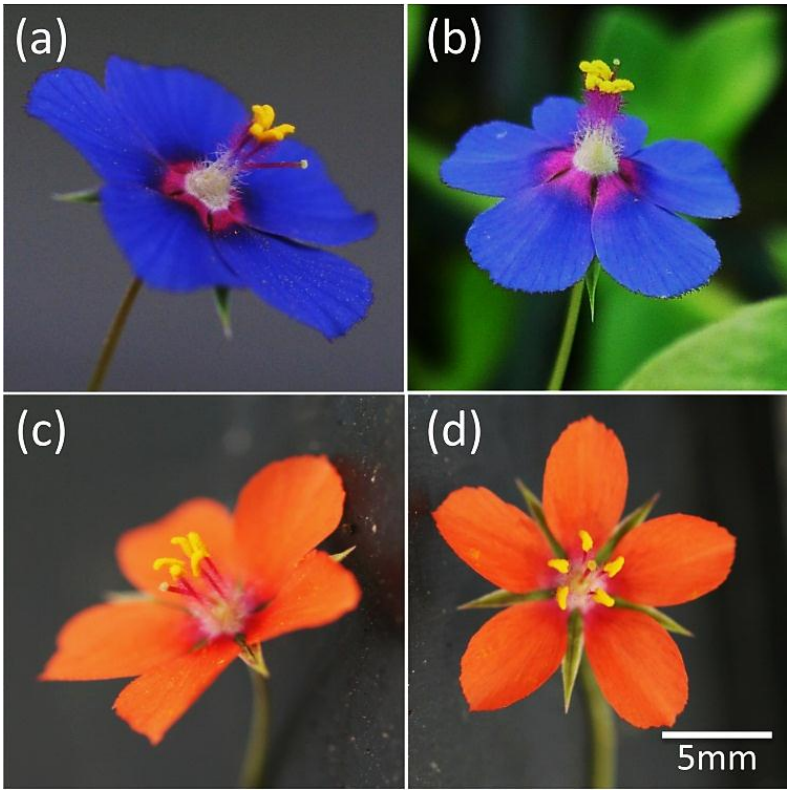

Figure 1. Flowers of the blue and red lineage of *Lysimachia arvensis* during the first (a, c) and second anthesis day (b, d).

Individuals of *L. arvensis* also vary discretely in the colour of their flowers, with two colour 'morphs' belonging to genetically divergent lineages. Most populations in Europe comprise only plants with red flowers, but plants with blue flowers are found in drier Mediterranean regions, either on their own, or mixed with red-flowered plants (Arista et al., 2013). In mixed blue-biased populations, the most common in the Mediterranean, both blue- and red-flowered plants are visited mainly by the same solitary bees, which have a clear preference for the blue plants (Ortiz et al., 2015). Moreover, both red- and blue-flowered plants show higher inbreeding coefficients in mixed populations, indicating a certain level of isolation between the two flower colours (F. Jiménez-López et al., unpub. results). Blue- and red-flowered plants can be crossed to produce fully fertile progeny with flowers of intermediate colour (Jiménez-López, unpub. results). However, variation in colour in natural populations is largely discrete, with F_1 progeny of parents with different flower colours being uncommon, and F_2 progeny resulting from experimental crosses having reduced fitness (Jiménez-López et al., unpub. results). Thus, although current taxonomy recognises a single species of *L. arvensis* (Pujadas, 1997; Manns & Anderberg, 2009), it seems likely that individuals with different flower colours belong to different evolutionary lineages and are incipient species, with somewhat different habitat preferences and a modest degree of post-zygotic genetic isolation. The almost total absence of F_1 hybrid individuals with intermediate salmon-coloured flowers in mixed populations (Jiménez-López et al., unpub. results) also points to an effective degree of pre-zygotic isolation. In this article, we refer to the flower 'morphs' as different lineages, in recognition of the emerging evidence for their evolutionary divergence.

Given both the lower pollinator attendance to the red lineage of *L. arvensis* in mixed populations (Ortiz et al., 2015) and the tendency towards lower fitness for progeny of inter-morph crosses (Jiménez-López et al. unpub. results), a divergence between morphs should be expected to reduce competition for shared resources (i.e., pollinators) via ecological character displacement (Slatkin, 1980; Schluter, 2000) or to alleviate the cost of reproductive interference via reproductive character displacement (Pfennig & Pfennig, 2009). Traits promoting autonomous selfing might thus be selected as a way to decouple reproduction from pollinator services, thus providing reproductive assurance and avoiding interspecific pollen transfer (Levin 1971; Fishman & Wyatt, 1999). Given that blue flowers are much more abundant than red flowers in mixed populations, the blue lineage should have a stronger negative effect on the red lineage than the converse. Accordingly, we hypothesised that the red lineage should show a more enhanced capacity for self-pollen deposition than the blue lineage in mixed populations. This hypothesis should hold not only in terms of reduced herkogamy, but also, and in particular, in terms of reduced herkogamy in first-day flowers, with greater

early autonomous self-pollen deposition and thus a tendency towards prior selfing. In contrast, we hypothesised that the red lineage would show greater herkogamy and more delayed pollen deposition in pure red populations, similar to the blue lineage. To test these hypotheses, and to assess the relative effectiveness of lateral versus vertical herkogamy, and of approach versus reverse herkogamy, we assessed the relationship between floral trait variation and the capacity for autonomous selfing in the absence of pollinators. We also recorded variation in herkogamy for individual flowers for a total of 25 mixed- and pure-colour populations of *L. arvensis* to test the hypothesis of reproductive character displacement.

MATERIAL & METHODS

Study populations

We measured flowers in a total of 25 populations of *L. arvensis* in both Mediterranean and non-Mediterranean regions of Europe (Table S1). Populations were visited during the peak of flowering, and each was categorized as pure blue-flowered, pure red-flowered, or mixed (red- and blue-flowered plants in the same population). We sampled nine pure-blue, six pure-red and ten mixed populations, with 475 blue- and 430 red-flowered plants sampled in total. Sample sizes varied among populations (from ten to 35 individuals per lineage) because of variation in population size.

Measuring herkogamy traits

We measured traits implicating herkogamy in *L. arvensis* to assess their role in preventing self-pollination, and to assess variation across populations. All measurements were taken from fresh flowers, starting on their first day of anthesis, and based on photographs taken directly in the field or the glasshouse. We measured the two components of herkogamy in one flower per plant using the software ImageJ. Lateral herkogamy was measured as the angle between style and stamens (hereafter 'style-stamen angle'). We also measured the lengths of stamens and pistils (from the flower base to the centre of anthers or pistils), and we calculated the degree of herkogamy as the difference between pistil and stamen lengths (hereafter stigma-anther displacement).

The role of herkogamy in preventing autonomous self-pollen deposition

We measured self-pollen deposition for 59 plants of both the blue and red lineages in a glasshouse and thus from which pollinators were excluded. Plants were chosen with a view to including a wide range of trait variation in our sample. To assess the effect of lateral herkogamy on self-pollen deposition, we sampled a single flower from each

plant at the end of its first day of anthesis (1st-day flower). To assess the effect of approach or reverse herkogamy on self-pollen deposition, we sampled a second flower from each plant of its second day of anthesis (2nd-day flower). We stained stigmas from these flowers with aniline blue (Martin, 1959), and counted the number of pollen grains on them under a fluorescence microscope.

Data analysis

We used cubic regression analyses to assess the importance of lateral herkogamy in preventing self-pollen deposition. Self-pollen deposited on stigmas of 2nd-day flowers is the sum of pollen depositions during first and second day of anthesis. To ascertain exclusively the importance of stigma-anther displacement in preventing self-pollen deposition, we selected 2nd-day flowers whose angle during the previous day was greater than 20 degrees. This selection was based on the results of the cubic regression obtained for 1st-day flowers, which showed that self-pollen deposition is practically null when lateral herkogamy is over 20 degrees (see Results). Quadratic regression was then performed between self-pollen deposition and stigma-anther displacement for these 2nd-day flowers.

Pearson correlations between all the measured floral traits were calculated for each colour morph. To assess herkogamy trait variation among populations and between the blue and red lineages, we performed two-way ANOVAs, with population and colour treated as fixed effects with their interaction. To test whether the two types of herkogamy differed between pure and mixed populations, and whether these differences were affected by colour, we performed a multivariate analyses of variance (MANOVA), using both herkogamy measurements, with colour, population type (pure/mixed) and their interactions as fixed effects. Subsequently, two-way ANOVAs were carried out for each herkogamy trait to determine whether co-occurrence of lineages affected floral morphology. Here, colour, population type (pure or mixed) and their interaction were used as explanatory variables in the model.

Finally, we determined whether self-pollen deposition among red and blue flowered plants growing in pure and mixed populations depended on the two herkogamy traits. To do this, we calculated predicted self-pollen deposition by using the regression curves, previously obtained, between self-pollen deposition and each herkogamy trait for both pure and mixed populations. We used GLMs to gest for differences in predicted self-pollen deposition as a function of stigma-anther angle and displacement, flower colour, and type of population, with a Poisson error distribution and a log link function.

RESULTS

Variation in herkogamy across populations

Lateral style-stamen angle varied significantly between the blue and red lineages and among populations (interaction colour-by-population, $F_{8,871} = 2.185$, p = 0.027; Figs. 2a, 2c, 2e). In general, blue flowers had smaller angles than red ones in most populations (Fig. 2e). Flowers with angles > 20 degrees were found in 59% and 96% of blue- and

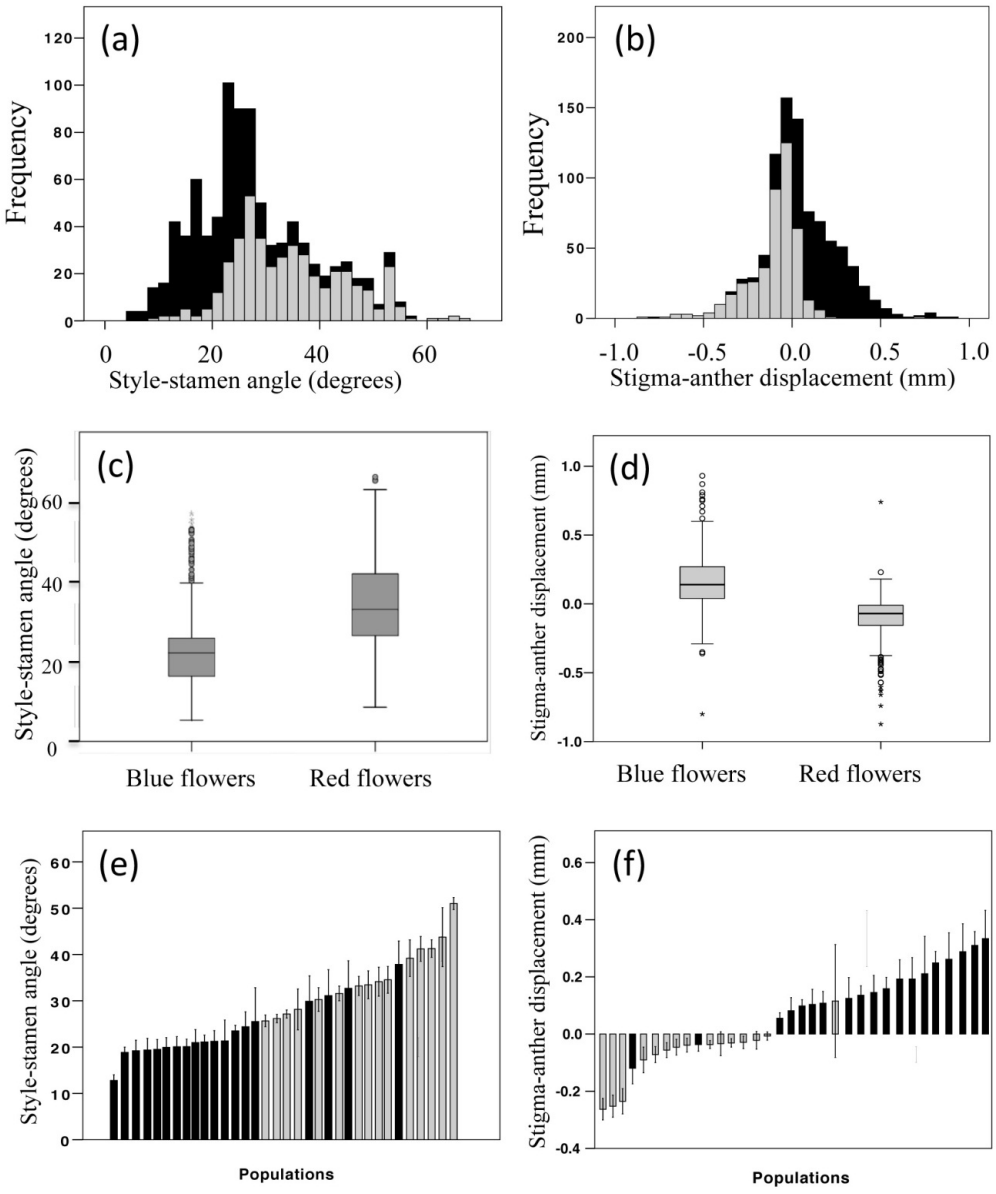

Figure 2. Frequency distribution of style-stamen angle in flowers in 1st day of anthesis (a) and stigma-anther displacement in flowers in 2nd day of anthesis (b). Differences between blue and red plants in the style-stamen angle (c) and in stigma-anther displacement (d). Medians, quartiles and ranges of the overall data are shown. Means and standard errors of the style-stamen angle (e) and in stigma-anther displacement (f) in each sampled population. In a, b, e and f, blue- and red-flowered plants are denoted in black and grey, respectively. Sample sizes: 475 blue flowers from 19 populations and 430 red flowers from 16 populations.

red-flowered plants, respectively (Fig. 2a). Vertical stigma-anther displacement also varied significantly between lineages and among populations (colour-by-population interaction $F_{8,871} = 3.91$, $p < 0.001$; Figs. 2b, 2d, 2f). In most populations, blue flowered plants were approach-herkogamous, while red flowers were predominantly reverse-herkogamous or had stigmas and anthers at the same level (Fig. 2f). In fact, blue flowers showed reverse herkogamy in only two populations, and in only one population did red flowers show approach herkogamy (Fig. 2f). Overall, approach herkogamy was found in 83.8% of blue flowers, but only in 17.7% of red ones. In blue-flowered plants, lateral style-stamen angle correlated significantly with vertical stigma-anther displacement ($R = 0.255$, $p < 0.001$), i.e., the larger the style-stamen angle, the greater the degree of approach herkogamy. In red-flowered plants, that correlation was also significant but negative ($R = -0.11$, $p = 0.018$), i.e., the larger the style-stamen angle, the greater the degree of reverse herkogamy.

MANOVA results showed marked and significant differences in flower morphology between blue- and red-flowered plants in pure and mixed populations, with a significant interaction between these two factors (Table 1). Flower colour was particularly strongly

Table 1. (a) MANOVA between two herkogamy traits: style-stamen angle and stigma-anther displacement as dependent variables and lineage (red or blue), population type (pure or mixed) and their interaction as independent variables. (b) Two-way ANOVAS between each of the two herkogamy traits and lineage, population type and their interaction.

Effect	λ Wilk	F	df	P
(a) MANOVA				
Intercept	0.103	3919.39	2	<0.000
Lineage	0.459	529.83	2	<0.000
Population type	0.939	29.40	2	<0.000
Lineage x Population type	0.952	22.79	2	<0.000
(b) Two-way ANOVAs				
Style-stamen angle				
Intercept		7806.53	1	<0.000
Lineage		346.73	1	<0.000
Population type		30.783	1	<0.000
Lineage x Population type		0.179	1	0.672
Stigma-anther displacement				
Intercept		18.957	1	<0.000
Lineage		589.02	1	<0.000
Population type		21.05	1	<0.000
Lineage x Population type		44.10	1	<0.000

associated with floral morphology (Table 1). Interestingly, flowers of both the blue and red lineages had a smaller lateral style-stamen angle in mixed than in pure populations, but stigma-anther displacement only differed in the red lineage. In mixed populations, the stigmas of the red lineage were situated at almost the same level as the anthers, whereas in pure populations they showed marked reverse herkogamy (Table 1, Fig. 3).

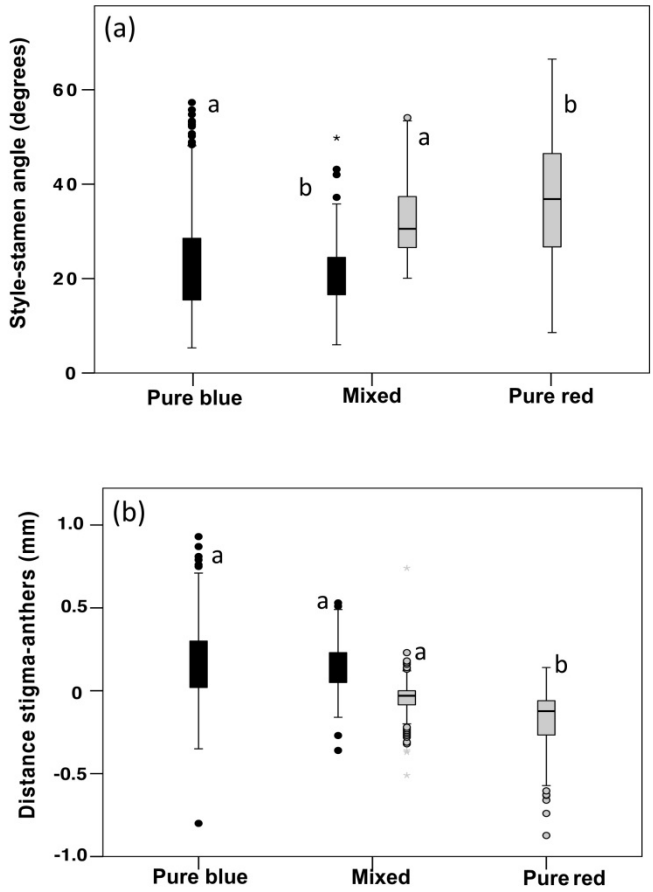

Figure 3. Style-stamen angle (a) and stigma-anther displacement (b) of blue and red lineages of *L. arvensis* in pure and mixed populations. Median, quartiles, maximum and minimum are shown. Different letters indicate significant differences for the trait between pure and mixed populations of each lineage.

The role of herkogamy traits in preventing autonomous self-pollination

Self-pollen deposition varied non-linearly with both measures of herkogamy, with lateral herkogamy particularly effective at reducing self-pollen deposition. Specifically, self-pollen deposition decreased from 15 pollen grains for 1st-day flowers with angles of about 10 degrees to effectively zero for those flowers with angles > 20 degrees (Fig. 4). Vertical stigma-anther displacement for 2nd-day flowers was also effective in preventing self-pollen deposition, with the highest pollen deposition observed for flowers in which stigma and anthers were at the same level (Fig. 4). The lowest self-pollen deposition was found in flowers with approach herkogamy: the number of pollen grains dropped

dramatically from a mean of 25 pollen grains for stigmas protruding 0.01 mm above the stamens, to a mean of only 6 pollen grains for stigmas protruding 0.5 mm. Reverse herkogamy was much less efficient in preventing self-pollen deposition: self-pollen deposition was only slightly lower for flowers with stigmas 0.8 mm below the anthers (mean of 15 pollen grains) than for flowers with stigma and anthers at the same level (mean of 21 pollen grains, Fig. 4).

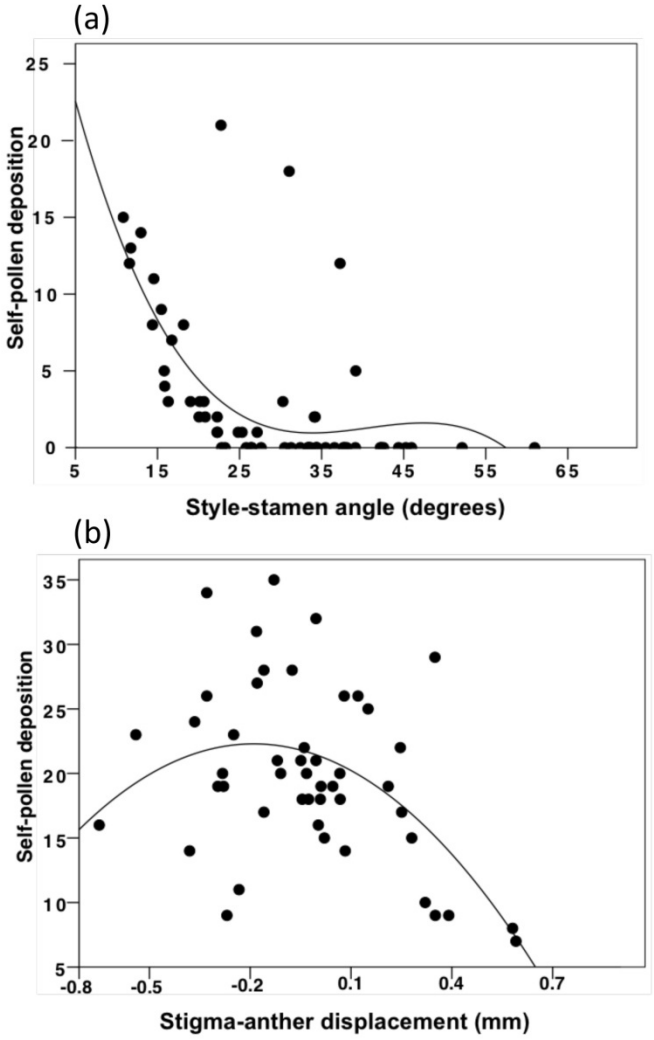

Figure 4. Relationship between style-stamen angle and self-pollen deposition in flowers on the 1st day of anthesis (a), and relationship between stigma-anther displacement and self-pollen deposition in flowers on the 2nd day of anthesis (b) in *L. arvensis*.

Differences in lateral herkogamy between pure and mixed populations affected the predicted self-pollen deposition only in the red lineage, in which a decrease was found in mixed populations (colour-by-population interaction: Wald chi-square = 12.21, 1 df, p = 0.000; Fig. 5). In contrast, differences in vertical herkogamy significantly affected the predicted self-pollen deposition only in the blue lineage, in which an increase was

found (colour-by-population interaction: Wald chi-square = 10.47, 1 df, p = 0.001; Fig. 5).

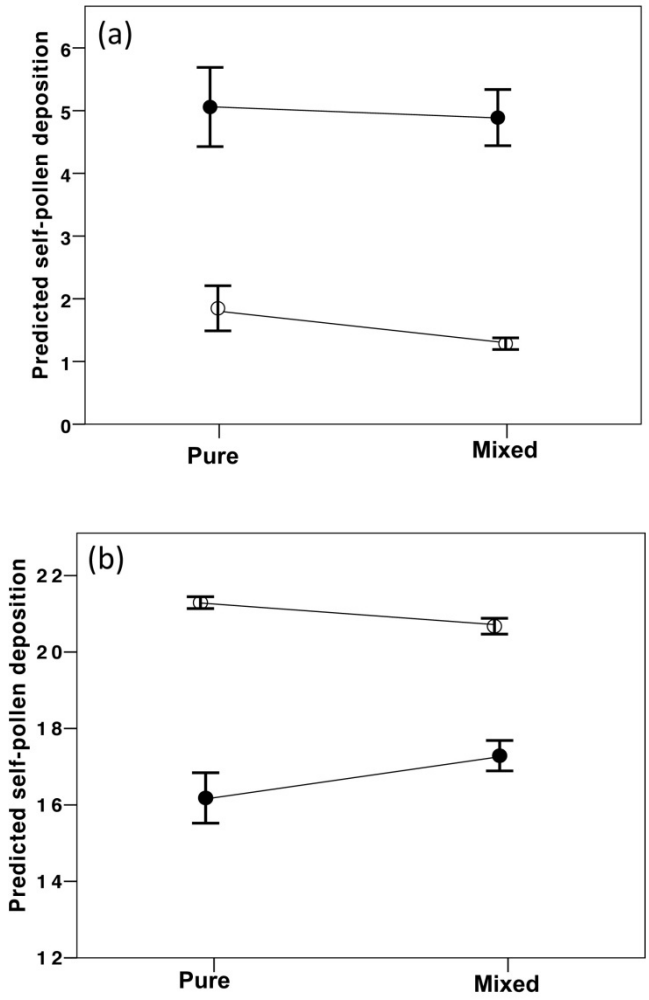

Figure 5. Predicted self-pollen deposition according to style-stamen angles (a) and stigma-anther displacement (b) in pure and mixed populations of *L. arvensis*. The blue and red lineages are denoted in black and grey, respectively.

DISCUSSION

Lysimachia arvensis showed two types of herkogamy that changed during the life of its flowers. During the first day, flowers displayed lateral herkogamy, where the style is displaced laterally from the central axis, forming an angle with the stamens. During the second day, the style moved upright and, depending on its length, came to be positioned below or above the anthers, showing vertical herkogamy. Although both types of herkogamy reduced autonomous self-pollination (see below), lateral herkogamy proved to be more effective, with no self-pollen deposition observed for flowers with an angle > 20 degrees. Given that most flowers had angles > 20 degrees,

this suggests that, in the field, autonomous self-pollination is not possible during the first day of anthesis in these flowers during the lateral herkogamous phase.

The effectiveness of lateral herkogamy in *L. arvensis* may be related to its pollination biology. *Lysimachia arvensis* offers only pollen as a reward to pollinators. Solitary bees, its main pollinators (Ortiz et al., 2015), manipulate the anthers to collect pollen. A marked lateral separation between style and anthers would likely limit contact between them even when pollinators collect pollen, avoiding interference between the sexes and improving cross-pollination during the first day of anthesis. Thus, flowers with wider angles depend more strongly on pollinator activity for pollination during the first day of anthesis. After flower closure at the end of the first day of anthesis, however, the style-stamen angle diminishes, so that, on the second day, opportunities for outcrossing versus selfing depend no longer on lateral stigma displacement but rather on the vertical position of the stigma above or below the anthers. At this stage, self-pollination occurs mainly in flowers with stigmas at the same level of the anthers, and more in flowers with reverse that with approach herkogamy.

Differences in the role of approach and reverse herkogamy in controlling self-pollen deposition have been suggested previously (Webb & Lloyd, 1986; Barrett & Shore, 1987; Barrett, 2003). In species with stigmas placed always above the anthers, it is common that outcrossing increases with herkogamy (Brunet & Eckert 1998; Herlihy & Eckert, 2007; but see Medrano et al., 2005). However, the role of herkogamy when it ranges from reverse to approach has been much less studied and would not necessarily reduce selfing (Kulbaba & Worley, 2008). Our results for *L. arvensis* are in accordance with those for *Datura stramonium* (Motten & Stone 2000) and *Gilia achilleifolia* (Takebayashi et al., 2006), in which outcrossing is favoured only when the stigma is held above the anthers, but not when it is below them. In *L. arvensis*, flowers with stigmas at the same level of the anthers or below them received between 15-21 pollen grains by autonomous self-pollination. The stigmatic pollen load needed to ensure full seed-set ranges from 2 to 6 grains per ovule (Cruden, 1977; Shore & Barrett, 1984; Aizen & Harder, 2007), although pollen quality can also limit seed-set (Aizen & Harder, 2007). Since flowers of *L. arvensis* have 17-30 ovules (Arista et al., 2013; Ortiz et al., 2015), as much as half of the ovules could be fertilized. Thus, reverse herkogamy during the second-day of anthesis can confer reproductive assurance via delayed selfing (Lloyd & Shoen, 1992), although seed-set is not complete.

Differences in the expression of herkogamy between the blue and red lineages

The blue and red lineages of *L. arvensis* differed consistently in both herkogamy traits, a fact that clearly indicates a history of reproductive isolation between lineages. Red

flowers showed strong lateral herkogamy, with style-stamen angles generally > 20 degrees. This suggests that red flowers are totally dependent on pollinator activity during the first day of anthesis. However, during the 2nd day of anthesis, the great majority of red flowers showed no herkogamy, or reverse herkogamy, and there was thus a potential for autonomous delayed self-pollination. We sampled a large number of populations, and the fact that lateral herkogamy was consistently marked for the red lineage, and that no prior or competing selfing was found, suggests that it mainly outcrosses in the wild when pollinators are present.

The blue-flowered lineage appears to have a mating strategy that differs from that of the red-flowered lineage in being both more variable and more frequently susceptible to autonomous self-pollination. In the blue lineage, the degree of lateral herkogamy varied greatly, with flowers in about half of the studied plants and populations having stigma-stamen angles < 20 degrees. These flowers are likely susceptible to autonomous self-pollination already on the first day of anthesis. However, on the second day of anthesis, the great majority of blue flowers showed approach herkogamy, and flowers with greater angles of lateral herkogamy had also a greater degree of approach herkogamy subsequently. Thus, while blue-flowered plants with angles of lateral herkogamy > 20 degrees appear to be incapable of autonomous self-pollination throughout their life, those with angles < 20 degrees (which display lower approach herkogamy too) likely self-pollinate throughout anthesis via competing selfing (Lloyd 1979; Leclerc-Potvin & Ritland 1994). The blue lineage thus shows a mixed mating system, with selfing rates that probably vary more than those of the red lineage.

Although classical population genetic models for the evolution of plant mating systems concluded that only complete selfing or complete outcrossing should be evolutionarily stable (Lande & Schemske, 1985; Jarne and Charlesworth, 1993; Holsinger, 1996), empirical studies indicate that more than one third of the flowering plants show mixed reproductive systems (Goodwillie et al., 2005; Johnston et al., 2009) and recent models that incorporate ecological factors such as cross-pollen availability, gamete discounting (Johnston et al, 2009) or temporal (Cheptou & Schoen, 2002) and spatial variability in the expression of inbreeding depression (Pujol et al., 2009) help to account for these cases. The variation in herkogamy traits of blue-flowered individuals of *L. arvensis* hints at possible differences in the ecological context of their mating, such that, for instance, some populations of the blue lineage may be subject to more frequent cross-pollen limitation due to selection for reproductive assurance, while others have evolved in habitats with high pollinator attendance. Such hypotheses await testing. The variation in herkogamy traits found in the blue lineage might also reflect spatial variability in the expression of inbreeding depression (Pujol et al., 2009).

Variation in herkogamy traits in mixed versus pure populations

We found that the degree of herkogamy in *L. arvensis* was lower in mixed populations of the red and blue lineages than in pure populations, particularly for lateral herkogamy. We hypothesised that this pattern would be particularly evident for the red lineage, which is less abundant than the blue lineage in mixed populations and which might therefore stand to gain more in avoiding between-lineage crosses because of the strong frequency dependence of selection expected for reproductive character displacement (Cooley, 2007; Brys et al., 2014). Although reduced lateral herkogamy was observed in mixed populations for both lineages, and not just the red lineage, contrary to our predictions, vertical herkogamy was significantly lower only for the red lineage in mixed populations. These observations are thus partially in line with our predictions, but their functional significance remains untested. A quantitative genetic analysis of variation in herkogamy for both lineages would be valuable, as would a direct test of the hypothesised link between herkogamy traits and within- versus between-lineage mating. However, our finding of higher inbreeding coefficients for both lineages in mixed populations is broadly consistent with expectations under a model invoking character displacement. The evolution towards selfing has been interpreted as a mechanism to avoid interference between closely co-occurring species of a number of other taxa, including as *Phlox* (Levin, 1985), *Solanum* (Whalen 1978), *Arenaria* (Fishman & Wyatt, 1999), *Centaurium* (Brys et al., 2014), *Mimulus* (Grossenbacher & Whittall, 2011) or *Clerodendrum* (Miyake & Inoue, 2003). It seems unlikely that an increased capacity for selfing in *L. arvensis* in mixed population has evolved simply as a result of greater benefits for reproductive assurance at sites with both lineages than in those with only one.

Our study has shown that both herkogamy traits of *L. arvensis* not only control the amount of self-pollen deposited on stigmas, but also when that deposition occurs. The high variability in both herkogamy traits suggests differences in mating systems among populations. A relationship between herkogamy and mating system has been reported for other species with either lateral (Brys & Jackemin, 2011) or vertical herkogamy (Motten & Stone, 2000; Takebayasi et al., 2006). However, in *L. arvensis* the presence in the flowers of two consecutive herkogamy traits, which could be subjected to different selective pressures, might constrain evolution towards complete selfing or complete outcrossing. This situation is further complicated by the fact that the correlation between herkogamy traits is positive in the blue lineage but negative in the red. We found individuals displaying low lateral herkogamy and reverse herkogamy, others with high lateral herkogamy and approach herkogamy, and yet others with high lateral herkogamy and reverse herkogamy. Of these three combinations, only the first implies predominant selfing, observed mainly in the blue lineage when co-occurring

with the red lineage. It is too early to conclude that differences in herkogamy between the two lineages in mixed populations have evolved to avoid reproductive interference by increasing selfing, but this is a possibility that deserves future attention.

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AUTHOR CONTRIBUTION

MA, PLO and MT planned and designed the research. FJJL collected all the data and made the measurements and the exclusion experiments; FJJL, MA and PLO analyzed the data. MA, PLO and JRP wrote the first versions of the manuscript that was later edited by all authors.

REFERENCES

Aguilar R, Ashworth L, Galetto L, Aizen MA. 2006. Plant reproductive susceptibility to habitat fragmentation: review and synthesis through a meta-analysis. *Ecology letters* **9**: 968-980.

Arista M, Talavera M, Berjano R, Ortiz PL. 2013. Abiotic factors may explain the geographical distribution of flower colour morphs and the maintenance of colour polymorphism in the scarlet pimpernel. *Journal of Ecology* **101**: 1613-1622.

Arista M, Berjano R, Viruel J, Ortiz MA, Talavera M, Ortiz PL. 2017. Uncertain pollination environment promotes the evolution of a stable mixed reproductive system in the self-incompatible Hypochaeris salzmanniana (Asteraceae). *Annals of botany*, **120**: 447-456.

Barrett SCH. 2002. The evolution of plant sexual diversity. *Nature Reviews Genetics* **3**: 274-284.

Barrett SCH. 2003. Mating strategies in flowering plants: the outcrossing–selfing paradigm and beyond. *Philosophical Transactions of the Royal Society B: Biological Sciences* **358**: 991-1004.

Barrett SCH, Shore JS. 1987. Variation and evolution of breeding systems in the Turnera ulmifolia L. complex (Turneraceae). *Evolution* **41**: 340-354.

Barrett SC, Jesson LK, Baker AM. 2000. The evolution and function of stylar polymorphisms in flowering plants. *Annals of Botany* **85**: 253-265.

Barrett SC, Lloyd DG, Arroyo J. 1996. Stylar polymorphisms and the evolution of heterostyly in Narcissus (Amaryllidaceae). In Lloyd DG, Barret SCH, eds. *Floral biology: Studies on Floral Evolution in Animal-pollinated Plants.* Springer, Boston, MA, 339-376.

Beans CM. 2014. The case for character displacement in plants. *Ecology and evolution* **4**: 862-875.

Brunet J, Eckert CG. 1998. Effects of floral morphology and display on outcrossing in blue columbine, Aquilegia caerulea (Ranunculaceae). *Functional Ecology*, **12**: 596-606.

Brys R, Broeck AV, Mergeay J, Jacquemyn H. 2014. The contribution of mating system variation to reproductive isolation in two closely related Centaurium species (Gentianaceae) with a generalized flower morphology. *Evolution* **68**: 1281-1293.

Brys R, Cauwenberghe J, Jacquemyn H. 2016. The importance of autonomous selfing in preventing hybridization in three closely related plant species. *Journal of Ecology* **104***:* 601-610.

Brys R, Jacquemyn H. 2011. Variation in the functioning of autonomous self-pollination, pollinator services and floral traits in three Centaurium species. *Annals of Botany* **107**: 917–925.

Brys R, Jacquemyn H. 2012. Effects of human-mediated pollinator impoverishment on floral traits and mating patterns in a short-lived herb: an experimental approach. *Functional Ecology*, **26**: 189-197.

Carvalheiro LG, Biesmeijer JC, Benadi G, Fründ J, Stang M, Bartomeus I, Baldock KC et al. 2014. The potential for indirect effects between co-flowering plants via shared pollinators depends on resource abundance, accessibility and relatedness. *Ecology letters*, **17**: 1389-1399.

Cheptou PO, Schoen DJ. 2002. The cost of fluctuating inbreeding depression. *Evolution* **56**: 1059-1062.

Cruden RW. 1977. Pollen-ovule ratios: a conservative indicator of breeding systems in flowering plants. *Evolution* **31**: 32-46.

Fisher RA. 1941. Average excess and average effect of a gene substitution. *Annals of Eugenics*, **11**: 53-63.

Fishman L, Wyatt R. 1999. Pollinator-mediated competition, reproductive character displacement and the evolution of selfing in Arenaria uniflora (Caryophyllaceae). *Evolution* **53**: 1723-1733.

Goodwillie C, Kalisz S, Eckert CG. 2005. The evolutionary enigma of mixed mating systems in plants: occurrence, theoretical explanations, and empirical evidence. *Annu. Rev. Ecol. Evol. Syst.***36**: 47-79.

Grossenbacher DL, Whittall JB. 2011. Increased floral divergence in sympatric monkeyflowers. *Evolution*, **65**: 2712-2718.

Harder LD, Routley MB. 2006. Pollen and ovule fates and reproductive performance by flowering plants. In Harder LD. Barrett SCH, eds. *Ecology and evolution of flowers.* Oxford University Press*,* Oxford, 61-80.

Herlihy CR, Eckert CG. 2002. Genetic cost of reproductive assurance in a self-fertilizing plant. *Nature* **416**: 320.

Herlihy CR, Eckert CG. 2007. Evolutionary analysis of a key floral trait in Aquilegia canadensis (Ranunculaceae): genetic variation in herkogamy and its effect on the mating system. *Evolution* **61**: 1661-1674.

Holsinger KE. 1996. Pollination biology and the evolution of mating systems in flowering plants. *Evolutionary Biology* **29**: 107–149.

Holsinger KE, Feldman MW, Christiansen FB. 1984. The evolution of self-fertilization in plants: a population genetic model. *The American Naturalist* **124**: 446-453.

Jarne P, Charlesworth D. 1993. The evolution of the selfing rate in functionally hermaphrodite plants and animals. *Annual Review of Ecology and Systematics* **24**: 441-466.

Jesson LK, Barrett SC. 2005. Experimental tests of the function of mirror-image flowers. *Biological Journal of the Linnean Society* **85**: 167-179.

Jiménez-López FJ, Arista M, Talavera M, Pannell JR, Ortiz PL. 2019. Heritabilities of lateral and vertical herkogamy in Lysimachia arvensis. *Plant Species Biology*, in press.

Kalisz S, Vogler DW, Hanley KM. 2004. Context-dependent autonomous self-fertilization yields reproductive assurance and mixed mating. *Nature* **430**: 884.

Kulbaba MW, Worley AC. 2008. Floral design in Polemonium brandegei (Polemoniaceae): genetic and phenotypic variation under hawkmoth and hummingbird pollination. *International Journal of Plant Sciences* **169**: 509-522.

Lande R, Schemske DW. 1985. The evolution of self-fertilization and inbreeding depression in plants. I. Genetic models. *Evolution* **39**: 24-40.

Leclerc-Potvin C, Ritland K. 1994. Modes of self-fertilization in *Mimulus guttatus* (Scrophulariaceae): a field experiment. *American Journal of Botany* **81**: 199-205.

Levin D. 1971. The origin of reproductive isolating mechanisms in flowering plants. *Taxon* **21**: 91-113.

Levin D. 1985. Reproductive character displacement in *Phlox*. *Evolution* **39**: 1275-1281.

Li QJ, Xu ZF, Kress WJ, Xia YM, Zhang L, Deng XB, Gao JY, Bai ZL. 2001. Pollination: flexible style that encourages outcrossing. *Nature* 410: 432.

Lloyd DG. 1979. Some reproductive factors affecting the selection of self fertilization in plants. *American Naturalist* **113**: 67-79.

Lloyd DG. 1992. Self-and cross-fertilization in plants. II. The selection of self-fertilization. *International Journal of Plant Sciences* **153**: 370-380.

Lloyd DG, Schoen DJ. 1992. Self-and cross-fertilization in plants. I. Functional dimensions. *International Journal of Plant Sciences* **153**: 358-369.

Lloyd DG, Webb CJ. 1992. The evolution of heterostyly. In: Barrett S.C.H. (eds) *Evolution and Function of Heterostyly*. Monographs on Theoretical and Applied Genetics, v 15. Springer, Berlin, Heidelberg, 151-178.

Losos JB. 2000. Ecological character displacement and the study of adaptation. *Proceedings of the National Academy of Sciences* **97**: 5693-5695.

Manns U, Anderberg AA. 2005. Molecular phylogeny of *Anagallis* (Myrsinaceae) based on ITS, trn LF, and ndh F sequence data. *International Journal of Plant Sciences* **166**: 1019-1028.

Manns U, Anderberg AA. 2007. Relationships of *Anagallis foemina* and *A. arvensis* (Myrsinaceae): new insights inferred from DNA sequence data. *Molecular Phylogenetics and Evolution* **45**: 971-980.

Manns U, Anderberg AA. 2009. New combinations and names in *Lysimachia* (Myrsinaceae) for species of *Anagallis*, *Pelletiera* and *Trientalis*. *Willdenowia* **39**: 49-54.

Martin FW. 1959. Staining and observing pollen tubes in the style by means of fluorescence. *Stain technology* **34**: 125-128.

Martin NH, Willis JH. 2007. Ecological divergence associated with mating system causes nearly complete reproductive isolation between sympatric *Mimulus* species. *Evolution* **61**: 68-82.

Medrano M, Herrera CM, Barrett SC. 2005. Herkogamy and mating patterns in the self-compatible daffodil *Narcissus longispathus*. *Annals of Botany* **95**: 1105-1111.

Miyake T, Inoue K. 2003. Character displacement in style length between pollinator-sharing *Clerodendrum trichotomum* and *C. izuinsulare* (Verbenaceae). *Plant Systematics and Evolution* **243**: 31-38.

Moeller DA. 2006. Geographic structure of pollinator communities, reproductive assurance, and the evolution of self-pollination. *Ecology* **87**: 1510–1522.

Motten AF, Stone JL. 2000. Heritability of stigma position and the effect of stigma-anther separation on outcrossing in a predominantly self-fertilizing weed, *Datura stramonium* (Solanaceae). *American Journal of Botany* **87**: 339-347.

Opedal OH. 2018. Herkogamy, a Principal Functional Trait of Plant Reproductive Biology. *International Journal of Plant Sciences* **179**: 677-687.

Ortiz PL, Berjano R, Talavera M, Rodríguez-Zayas L, Arista M. 2015. Flower colour polymorphism in *Lysimachia arvensis*: How is the red morph maintained in Mediterranean environments? *Perspectives in Plant Ecology, Evolution and Systematics* **17**: 142-150.

Pfennig K, Pfennig D. 2009. Character displacement: ecological and reproductive responses to a common evolutionary problem. *The Quarterly Review of Biology* **84**: 253-276.

Pujadas AJ. 1997. *Anagallis*. In Castroviejo S et al. Flora iberica. Vol 5. Jardín Botánico de Madrid. CSIC, España. 57-62.

Pujol B, Zhou SR, Vilas JS, Pannell JR. 2009. Reduced inbreeding depression after species range expansion. *Proceedings of the National Academy of Sciences* **106**: 15379-15383.

Roels SAB, Kelly JK. 2011. Rapid evolution caused by pollinator loss in *Mimulus guttatus*. *Evolution* **65**: 2541-2552.

Schluter D. 2000. Ecological character displacement in adaptive radiation. *American Naturalist*, **156**: S4-S16.

Servedio MR, Noor MAF. 2003. The role of reinforcement in speciation: theory and data. *Annual Review of Ecology Evolution and Systematics* **34**: 339-364.

Shivanna KR. 2015. Reproductive assurance through autogamous self-pollination across diverse sexual and breeding systems. *Current Science* **109**: 1255-1263.

Shore JS, Barrett SC. 1984. The effect of pollination intensity and incompatible pollen on seed set in *Turnera ulmifolia* (Turneraceae). *Canadian Journal of Botany* **62**: 1298-1303.

Slatkin M. 1980. Ecological character displacement. *Ecology* **61**: 163-177.

Stebbins GL. 1957. Self fertilization and population variability in the higher plants. *American Naturalist* **91**: 337–354.

Subramaniam B, Rausher MD. 2000. Balancing selection on a floral polymorphism. *Evolution* **54**: 691-695.

Takebayashi N, Wolf DE, Delph LF. 2006. Effect of variation in herkogamy on outcrossing within a population of *Gilia achilleifolia*. *Heredity* **96**: 159-165.

Toräng P, Vikström L, Wunder J, Wötzel S, Coupland G, Ågren J. 2017. Evolution of the selfing syndrome: Anther orientation and herkogamy together determine reproductive assurance in a self-compatible plant. *Evolution* **71**: 2206-2218.

Vallejo-Marín M, Uyenoyama MK. 2004. On the evolutionary costs of self-incompatibility: incomplete reproductive compensation due to pollen limitation. *Evolution* **58**: 1924-1935.

Webb CJ, Pearson PE. 1993. The evolution of approach herkogamy from protandry in New Zealand *Gentiana* (Gentianaceae)*. Plant Systematics and Evolution* **186**: *187-191.*

Webb CJ, Lloyd DG. 1986. The avoidance of interference between the presentation of pollen and stigmas in Angiosperms. II. Herkogamy*. N.Z. J. Bot.* **24***: 163–178.*

Widmer A, Lexer C, Cozzolino S. 2009. Evolution of reproductive isolation in plants. *Heredity* **102**: 31.

SUPPORTING INFORMATION

Table S1. Herkogamy values (mean + standard deviation), sample size and geographic coordinates of pure blue, pure red and mixed populations of *Lysimachia arvensis*.

Populations	Coordinates	Type	Lineage
CHE. Chéserex. Les Rouges	46°24'11.6"N 6°09'34.8"E	P	Red
ESP. Alicante. Albaida	38°48'38.2''N 0°30'0.0''W	P	Blue
ESP. Alicante. Sierra de Bernia	38°40'19.4''N 0°2'45.8''W	P	Blue
ESP. Cádiz. Zahara de la Sierra	36°49'30.2"N 5°22'44.9"W	M	Blue & Red
ESP. Cádiz. Zahara de los Atunes	36°7'7.0"N 5°49'58.8"W	P	Blue
ESP. Castellón. Vinarós	40°30'49''N 0°30'34''W	P	Red
ESP. Cordoba. Bujalance	37°53'32.6"N 4°22'33.6"W	P	Blue
ESP. Gran Canaria. Almatriche	28°4'0.12''N 15°28'0.1''W	P	Blue
ESP. Huelva. Aracena	37°54'13.3"N 6°34'08.2"W	M	Blue & Red
ESP. Huelva. Aracena	37°54'44.8"N 6°34'08.0"W	M	Blue & Red
ESP. Huelva. Niebla	37°29'58.1''N 6°42'30.2''W	P	Blue
ESP. La Gomera	28º7'27.19''N 17º15'9.1''W	P	Blue
ESP. Sevilla	37°24'46.9"N 5°59'48.8"W	M	Blue & Red
ESP. Sevilla. Dos Hermanas	37°21'09.8"N 5°56'23.2"W	M	Blue & Red
ESP. Tarragona. Mont-Roig del Camp	42°04'43.3"N 2°09'07.7"E	M	Blue & Red
ESP. Tenerife. Anaga	28°33'01.5"N 16°12'04.6"W	P	Blue
ESP. Tenerife. La Orotava	28°22'42.3"N 16°30'36.1"W	P	Red
ESP. Toledo. Ocaña	39°57'40''N 3°31'59''W	P	Blue
FRA. Corsica. Solenzara	41°51'19.5''N 9°21'43.67''E	P	Red
MAR. Tetuan. Aouchtam Bni Said	35°29'36''N 5°8'39''W	M	Blue & Red
PRT. Alentejo. Sines	37°57'11''N 8°55'51''W	P	Red
PRT. Algarve. Albufeira. Praia de Falesia	37°5'12''N 8°10'19''W	P	Blue
PRT. Algarve. Carrapateira. Monte Clerigo	37°18'35.3"N 8°48'37.9"W	M	Blue & Red
PRT. Algarve. Carrapateira. Pont de Carrapateira	37°11'47.4"N 8°54'27.4"W	M	Blue & Red
TUN. Tabarka	36°57'46"N 8°44'51"E	P	Red

7. Estudio de barreras pre y postcigóticas entre linajes con diferente color floral en *Lysimachia arvensis* (L.) U. Manns & Anderb.

Jiménez-López F.J., Arista M. y Ortiz P.L.

(Jiménez-López F.J., Arista M. y Ortiz P.L. Estudio de barreras pre y postcigóticas entre linajes con diferente color floral en *Lysimachia arvensis* (L.) U. Manns & Anderb. En preparación)

RESUMEN

La especiación es un proceso continuo que implica divergencia fenotípica y genotípica, disminución del flujo génico y la presencia de barreras de aislamiento reproductivo entre linajes. Sin embargo, la separación de especies independiente requiere que las barreras de aislamiento reproductivo se mantengan en el tiempo. Tradicionalmente, las barreas de aislamiento reproductivo se dividen en precigóticas y postcigóticas. Entre las barreras precigóticas en plantas se encuentran las diferencias ecológicas, fenológicas, aislamiento por polinizadores o precedencia polínica, mientras que, entre las postcigóticas, la inferioridad ecológica y menor éxito reproductivo del híbrido, o la disminución de su fecundidad son las más comunes. El presente trabajo pretende determinar el grado de aislamiento reproductivo entre dos linajes de *Lysimachia arvensis* que difieren en el color de sus flores. Para ello se evalua la importancia de las barreras pre- y postcigóticas. Barrera geográfica mediante el estudio de nicho ambiental, fenológica con dos niveles diferentes, la dirigida por los polinizadores y la precedencia del polen entre las precigóticas; y la viabilidad de los híbridos, su supervivencia y su fertilidad entre las postcigóticas. Además, se ha cuantificado la viabilidad de la F_2 híbrida. El conjunto de resultados obtenidos en este trabajo demuestra que los dos linajes de *L. arvensis* muestran un alto grado de aislamiento reproductivo total, tanto teniendo en cuenta la distribución natural completa de la especie como considerando únicamente el área donde ambos linajes coexisten. Este aislamiento total se debe a la actuación consecutiva de las distintas barreras estudiadas, cada una de ellas con una contribución asimétrica al aislamiento reproductivo de cada linaje. En el linaje azul nuestros resultados mostraron un valor de aislamiento reproductivo total superior al 72 %, con gran diferenciación cuando este linaje actuaba como madre (RI_{total} = 0,8324) o como padre (RI_{total} = 0,5603). Por otro lado, para el linaje rojo encontramos un aislamiento superior al 85%, también con asimetría cuando actúa como madre (RI_{total} = 0,7648) y como padre (RI_{total} = 0,9114). Además, se observó que en la descendencia de segunda generación hubo una reducción significativa en la tasa de fructificación y en la producción de semillas tanto en plantas híbridas como en plantas originadas por retrocruces con los linajes parentales. En general, las barreras precigóticas no afectadas por la coocurrencia (polinizadores y precedencia polínica), fueron las más relevantes para *L. arvensis*, seguidas de las precigóticas afectadas por la coocurrencia, si no tenemos en consideración el aislamiento reproductivo generado por la distribución geográfica de cada linaje de la especie. Aunque el aislamiento no fue total, si que se ha observado un fuerte efecto de las barreras al flujo génico, especialmente en el linaje rojo. Y

quizás *L*. *arvensis* pueda ser definido como un modelo de especiación incipiente, donde el efecto de las barreras aquí analizadas está contribuyendo a una diferenciación genética.

Palabras clave: Aislamiento reproductivo, polimorfismo de color, flujo génico, especiación incompleta, especiación simpátrica.

INTRODUCCIÓN

La especiación es un proceso continuo que conlleva la divergencia fenotípica y genotípica de dos linajes, la evolución de caracteres que disminuyen el flujo génico y la formación de barreras de aislamiento reproductivo entre esos linajes. La selección impuesta por ambientes heterogéneos puede conducir a la divergencia fenotípica de las poblaciones y, de hecho, la presencia de grupos de individuos con características diferenciales es común dentro de las especies (Mallet 2008). Sin embargo, esos grupos no llegan a separarse como especies independiente hasta que no se originan barreras de aislamiento reproductivo (Nosil 2012) que deben mantenerse en el tiempo para que las especies incipientes lleguen a ser entidades distintas (Coyne & Orr 2004).

Tradicionalmente, las barreas de aislamiento reproductivo se han dividido en dos grandes grupos según operen antes (precigóticas) o después de la singamia (postcigóticas). Entre las barreras precigóticas en plantas se encuentran algunas tan importantes como las diferencias ecológicas, fenológicas, aislamiento por polinizadores o precedencia polínica (Mayr 1942; Grant 1981; Howard 1999), mientras que, entre las postcigóticas, la inviabilidad del híbrido, la disminución de su fecundidad (Dobzhansky 1937; Muller 1942) o su inferioridad ecológica y menor éxito reproductivo suelen ser las más comunes (Rundle et al. 2000; Schluter 2000; Dobzhansky 1937; Mayr 1942). Las barreras postcigóticas han sido estudiadas con mayor frecuencia en las plantas dada la facilidad para estimarlas de forma experimental mediante la realización de cruces en invernadero y el seguimiento posterior de los híbridos. En contraste, las precigóticas son más raramente valoradas y su estudio generalmente se limita a una o dos de ellas. Sin embargo, las barreras de aislamiento no actúan de forma aislada, sino que lo hacen secuencialmente y en los pocos casos estudiados, las precigóticas suelen ser mucho más importantes que las postcigóticas (Ramsey et al. 2003, Rieseberg & Willis 2007; Lowry et al. 2008; Palma-Silva et al. 2011). El orden de aparición de esas barreras en el proceso de especiación es desconocido (Schemske 2000). Así, por ejemplo, la adaptación de linajes a hábitat diferentes o la fidelidad de los polinizadores origina una importante barrera de aislamiento precigótico que surge tempranamente en la separación de los linajes de una especie, sin embargo, a veces las barreras postcigóticas surgen de manera tardía como un mecanismo de “refuerzo” para evitar la formación de híbridos mal adaptados (Dobzhansky 1937; Noor 1997; Hopkins 2013; Hopkins & Rausher 2014).

En la mayoría de las especies el aislamiento reproductivo se produce por la acción de múltiples barreras de aislamiento, precigóticas y/o postcigóticas, que actúan secuencialmente (Ramsey et al. 2003; Nosil & Crespi 2006). Identificar el grado en que

las barreras individuales contribuyen al aislamiento total entre especies supone un gran reto para los biólogos evolutivos. Determinar la importancia de las distintas barreras es necesario para identificar las fuerzas principales favorecedoras de la especiación, y si esas barreras se mantendrán frente a la hibridación. Una barrera reproductiva puede considerarse importante si, actuando sola, es un impedimento fuerte para el flujo de genes. La importancia de una barrera también se puede considerar mediante la evaluación de su contribución al aislamiento total en relación con otras barreras.

A pesar de que un objetivo fundamental en los estudios de especiación es determinar la importancia relativa de las múltiples formas de aislamiento reproductivo que operan entre pares de especies (Ramsey et al. 2003; Coyne & Orr 2004; Nosil et al. 2005; Martin & Willis 2007; Cozzolino & Scopece 2008; Lorry et al. 2008), el número de estudios que las aborda es sorprendentemente pequeño y aún menos que examinen linajes divergentes recientes. De hecho, Baak et al. (2015) en una revisión reciente de este tema encontraron que solo 137 trabajos abordan el estudio de más de 3 barreras de aislamiento, y que menos de cinco estudiaban todas las barreras importantes, incluida la geográfica. De hecho, la inclusión de la barrera geográfica solo se ha abordado en 4 grupos de especies: *Costus* (Kay 2006); *Petunia* (dell'Olivo et al. 2011), *Clarkia* (Runquist et al. 2014) o *Mimulus* (Martin & Willis 2007; Sobel & Streinfeld 2015). La dificultad de estudiar algunas barreras como la geográfica, reside en obtener un conjunto de datos suficientemente representativo que permita calcular qué porcentaje del área de distribución de dos linajes ocurre en simpatría o en alopatría. En la actualidad, el uso de herramientas de modelado de nicho permite estimar el área de distribución de los linajes a partir de muestreos incompletos si existe un conocimiento de los requerimientos edáficos y climáticos de esos linajes (Baak et al. 2015). Pero si el estudio de múltiples barreras de aislamiento raramente se aborda, prácticamente son inexistentes los estudios que alcanzan a estudiar la viabilidad de la F_2.

El presente trabajo pretende determinar el grado de aislamiento reproductivo entre dos linajes de *Lysimachia arvensis* que difieren en el color de sus flores. La especie presenta un linaje de flores azules bien adaptado a condiciones de alta insolación y baja pluviosidad que es común en la Cuenca Mediterránea y otro de flores rojas mejor adaptado a climas más mésicos que se distribuye fundamentalmente por el centro y norte de Europa (Arista et al. 2013). Ambos linajes aparecen en poblaciones simpátricas en el Mediterráneo, aunque generalmente el linaje azul es predominante. En esas poblaciones simpátricas, las abejas solitarias, que son los principales polinizadores, visitan las flores de ambos linajes, aunque muestran preferencia por el azul (Ortiz et al. 2015). Dado que el cruce entre linajes origina plantas con flores de

color intermedio (Jiménez-López et al., Capitulo 2) que raramente aparecen en las poblaciones, es esperable que entre esos linajes se exista un bajo flujo génico debido a la existencia de barreras de aislamiento. El objetivo de este trabajo es evaluar la importancia de las barreras pre- y postcigóticas entre linajes de color de *L. arvensis*. Para ello se estudiará un alto número de barreras que incluyen la geográfica, la fenológica con dos niveles diferentes, la dirigida por los polinizadores y la precedencia del polen entre las precigóticas y la viabilidad de los híbridos, su supervivencia y su fertilidad entre las postcigóticas. Además, se ha cuantificado la viabilidad de la F_2 híbrida, algo muy poco estudiado en los estudios de aislamiento reproductivo.

MATERIALES Y MÉTODOS

Especie de estudio

Lysimachia arvensis (L.) U. Manns y Anderb. es una especie anual oriunda del Mediterráneo y Europa e introducida en el resto del mundo. La especie es hermafrodita, autocompatible y sus flores muestran movimientos násticos, con apertura de los pétalos en la mañana y cierre por la tarde. Las flores presentan dos tipos consecutivos de hercogamia, una lateral el primer día de apertura y otra vertical durante el segundo y tercer día (Jiménez-López et al. 2019). Los linajes difieren en los tipos de hercogamia lo que sugiere diferencias en los sistemas de cruzamiento (Jiménez-López et al., capitulo 6). El polen es la única recompensa para los polinizadores, ya que las flores no producen néctar (Gibbs & Talavera 2001; Raine & Chittka 2007). El fruto es una cápsula con numerosas semillas que se dispersan fundamentalmente por barocoria.

Barreras de aislamiento

Para establecer el aislamiento reproductivo total (RI de aquí en adelante) entre los dos linajes de *L. arvensis*, se estudiaron rasgos ecológicos y biológicos que actúan como barreras tanto pre- como postcigóticas. En estas últimas, el estudio alcanzó hasta el desarrollo de la segunda generación tras el cruce entre linajes. Para cuantificar el grado de aislamiento reproductivo, la importancia de las múltiples formas de aislamiento debe ser valorada de una forma equivalente. Tradicionalmente se han usado diferentes índices para estimar la importancia de cada una de las barreras (ej. Dobzhansky 1951; Coyne & Orr 1989; Ramsey et al. 2003; Lowry et al. 2008), que oscilan entre un valor de 0 cuando no existe aislamiento y un valor de 1, cuando el aislamiento es completo. En un trabajo reciente, Sobel & Chen (2014) han comparado todos ellos y proponen un índice unificado que refleja en mejor medida la magnitud de cada una de las barreras. El índice de Sobel & Chen (2014) está relacionado

directamente con el flujo génico, es equivalente entre las barreras de aislamiento reproductivo y además es simétrico, es decir, mide tanto el rango positivo (flujo génico entre linajes menor del esperado si estos se cruzaran al azar, es decir, sin barreras) como el negativo (flujo génico mayor del esperado en dichas condiciones) del aislamiento reproductivo. Con esta aproximación, los índices oscilan entre -1 y 1 y además los índices relativos de las distintas barreras individuales pueden combinarse para calcular el aislamiento reproductivo total. Esta aproximación se utilizará en la estima del aislamiento reproductivo de los linajes de color de *L. arvensis*, pero con pequeñas modificaciones para algunas de las barreras cuando sea necesario. Dado que con frecuencia las barreras son asimétricas entre linajes (Tiffin et al. 2001; Martin & Willis 2007), el valor de cada barrera se ha calculado separadamente para cada linaje; además, dado que tales asimetrías también se dan entre géneros (Sobel & Chen 2014; Sobel & Streisfeld 2015), para todas las barreras que no dependen de la coexistencia (o no) de los linajes en el espacio-tiempo también se ha calculado separadamente el valor de la barrera para cada linaje cuando éste actúa como padre y cuando actúa como madre.

Barreras precigóticas

Se consideró la magnitud de cinco barreras precigóticas: aislamiento geográfico, asincronía en la fenología estacional de la floración, asincronía en el ritmo diario de apertura floral, aislamiento mediado por polinizadores y precedencia polínica.

Aislamiento geográfico

Para valorar la importancia del aislamiento geográfico entre linajes se modeló el nicho ambiental (ENM) de cada uno de los linajes para evaluar sus distribuciones potenciales en las condiciones bioclimáticas actuales. Para ello se utilizaron datos de presencia de 547 localidades distintas del linaje azul y 558 del rojo, combinando muestreos propios y datos obtenidos desde GBIF (GBIF.org [28 junio 2017]) que proceden de colecciones de herbario de las cuales se pudo comprobar el color de las flores, bien por visualización directa del pliego de herbario o porque esta información estaba registrada en la base de datos. Por otro lado, se utilizó un conjunto de 19 capas de variables bioclimáticas (Tabla 1) obtenidas de WorldClim (www.worldclim.org) con una resolución de 30 segundos. En cada capa se recortó una sección que incluía la región mediterránea y zonas colindantes donde se ha descrito la presencia de la especie utilizando DIVA-GIS 7.5.0 (Hijmans et al. 2001).

Se analizaron las correlaciones entre las variables bioclimáticas y solo un conjunto reducido de variables no correlacionadas que mostraban mayor contribución al modelo en base al índice de jackknife fueron seleccionadas. El uso de todas las variables en

comparación con las del conjunto reducido no suponía un incremento significativo en los valores de la curva de ajuste al modelo (AUC). Para evaluar la distribución potencial de cada linaje mediante ENM se utilizó el algoritmo de máxima entropía implementado en MAXENT v. 3.4.1 (Phillips et al. 2018). Para ello, los datos de presencia se dividieron al azar en dos bloques: datos de entrenamiento (75%), utilizados para construir el modelo, y datos de prueba (25%) para testar la precisión del modelo. Para cada linaje se realizaron diez réplicas del modelo por cada proyección, con 500 iteraciones por réplica, utilizando las opciones preestablecidas de MAXENT v.3.4.1.

Tabla 1. Variables bioclimáticas utilizadas en la predicción del ENM de *Lysimachia arvensis*.

Variable	Descripción de la variable	Variables utilizadas en cada linaje	
		Azul	Rojo
BIO1	Temperatura media anual		
BIO2	Rango diurno medio (media mensual (temperatura máxima - temperatura mínima))	X	
BIO3	Isotermia ((BIO2 / BIO7) * 100)		X
BIO4	Temperatura estacional (DS*100)		
BIO5	Temperatura máxima del mes más cálido	X	X
BIO6	Temperatura mínima del mes más frio		
BIO7	Rango de temperatura anual (BIO5-BIO6)		X
BIO8	Temperatura media del trimestre más húmedo	X	
BIO9	Temperatura media del trimestre más seco		
BIO10	Temperatura media del trimestre más cálido	X	X
BIO11	Temperatura media del trimestre más frio		
BIO12	Precipitación anual	X	
BIO13	Precipitación del mes más lluvioso	X	X
BIO14	Precipitación del mes más seco		
BIO15	Precipitación estacional (Coef. De variación)		
BIO16	Precipitación del trimestre más húmedo		X
BIO17	Precipitación del trimestre más seco	X	X
BIO18	Precipitación del trimestre más cálido	X	
BIO19	Precipitación del trimestre más frío		X

Una vez estimada el área de distribución potencial de cada linaje de *L. arvensis* se calculó qué proporción de dicho área es compartida con el otro linaje utilizando DIVA-DIS 7.5.0. El valor de la barrera de aislamiento reproductivo impuesto por diferencias de distribución geográfica (**RI $_{\text{geográfico}}$**) se calculó de acuerdo con la ecuación RI_{4C}

propuesta por Sobel & Chen (2014) para barreras precigóticas que dependen de la coexistencia (o no) de los linajes:

$$\mathrm{RI}_{\text{geográfico}} = 1 - \left(\frac{S}{S+U}\right)$$

Los términos **S** y **U** representan las proporciones (en tanto por uno) de área compartida y no compartida, respectivamente. El valor de esta barrera se calculó de forma independiente para cada linaje.

Asincronía en la fenología estacional de la floración

Para valorar el aislamiento impuesto por una asincronía entre las fenologías estacionales de ambos linajes, se registró la fenología de la floración en 11 poblaciones naturales simpátricas en el sur de la Península Ibérica (Tabla 2). En cada población se realizaron diversos censos repartidos a lo largo del periodo de floración,

Tabla 2. Características de las poblaciones de *Lysimachia arvensis* donde se realizaron los estudios de fenología floral estacional.

Población	Localidad	Coordenadas	Nº de flores observadas	
			Azules	Rojas
ESP_CA_Gr1	Cádiz. Grazalema 1	36º47,257'N 5º21,063'W	4892	302
ESP_CA_Gr2	Cádiz. Grazalema 2	36º45,304'N 5º26,153'W	3775	463
ESP_CA_ZS1	Cádiz. Zahara de la Sierra 1	36º50,718'N 5º23,227'W	11910	1885
ESP_CA_ZS2	Cádiz. Zahara de la Sierra 2	36º49,504'N 5º22,749'W	18844	3351
ESP_H_Ar1	Huelva. Aracena 1	37º54,221'N 6º34,137'W	395	624
ESP_H_Ar2	Huelva. Aracena 2	37º54,746'N 6º34,134'W	399	478
ESP_H_Ar3	Huelva. Los Marines	37º53,923'N 6º37,525'W	1477	829
ESP_H_Ar4	Huelva. Cortelazor alto	37º55,875'N 6º37,465'W	974	94
ESP_H_Hin	Huelva. Hinojos	37°17,01'N 6°23,852'W	12129	644
ESP_SE_Al	Sevilla. Santiponce	37°24,789'N 5°59,809'W	1369	5226
ESP_SE_DH	Sevilla. Dos Hermanas	37°21,091'N 5°56,225'W	5696	2073

registrándose mediante conteo directo el número total de flores abiertas de cada linaje. Dado que el promedio de vida de una flor es similar en ambos linajes (tres días), asumimos que todas las flores tenían la misma probabilidad de cruzamiento. Para calcular el valor del aislamiento debido a desfases en la fenología estacional (**$RI_{fenológico}$**) se utilizó la ecuación RI_{4S2} propuesta por Sobel and Chen (2014), que incorpora términos para controlar diferencias en abundancia relativa de los linajes:

$$\mathbf{RI}_{\mathbf{fenológico}} = \mathbf{1} - \mathbf{2} \times \left[\frac{\frac{\sum_i \left(\frac{A_i}{A_{total}} \times \frac{B_i}{A_i + B_i} \right)}{B_{total} / (A_{total} + B_{total})}}{\frac{\sum_i \left(\frac{A_i}{A_{total}} \times \frac{B_i}{A_i + B_i} \right)}{B_{total} / (A_{total} + B_{total})} + \frac{\sum_i \left(\frac{A_i}{A_{total}} \times \frac{A_i}{A_i + B_i} \right)}{A_{total} / (A_{total} + B_{total})}} \right]$$

Esta ecuación permite valorar una barrera de aislamiento temporal para un linaje A con respecto a otro linaje B considerando no solo las proporciones de tiempo disponible para el cruzamiento compartido y no compartido, sino también los valores de abundancia relativa de cada linaje en los distintos momentos de muestreo, así como en el cómputo global. En el caso que nos ocupa, los distintos términos de esta ecuación representan lo siguiente:

- "$\mathbf{A_i / A_{total}}$" representa la proporción de flores del linaje A (para el que estamos calculando el valor de la barrera) disponibles en el día de muestreo i con respecto al total de flores de ese linaje computadas a lo largo de la estación.

- "$\mathbf{A_i / (A_i + B_i)}$" representa la proporción de flores del linaje A (para el que estamos calculando el valor de la barrera) disponibles el día i con respecto al total de flores de ambos linajes ese mismo día.

- "$\mathbf{B_i / (A_i + B_i)}$" representa la proporción de flores del otro linaje (B) disponibles el día i con respecto al total de flores de ambos linajes ese mismo día.

- "$\mathbf{A_{total} / (A_{total} + B_{total})}$" y "$\mathbf{B_{total} / (A_{total} + B_{total})}$" indican qué proporción representan las flores del linaje A y del linaje B, respectivamente, registradas durante la estación con respecto al total registrado para ambos linajes.

Usando la mencionada ecuación se calculó el valor de esta barrera para cada linaje en cada una de las poblaciones estudiadas, y después se promediaron los valores de las distintas poblaciones para obtener un valor medio de aislamiento fenológico estacional ($\mathbf{RI_{fenológico}}$) para cada linaje.

Asincronía en el ritmo diario de apertura floral

El ritmo diario de antesis se estudió en tres poblaciones naturales en Sevilla (España) donde ambos linajes son simpátricos y en 3 rodales experimentales con ambos linajes. En cada población o rodal experimental se registró el ritmo de antesis floral durante diez días repartidos a lo largo del periodo de floración. Al inicio de cada día se seleccionaron al azar 100 botones florales de cada linaje antes de su apertura, y cada hora, entre las 8 a.m. y las 8 p.m., se registró el número de flores en antesis. Para cada población o rodal se promediaron los datos registrados durante los diez días de muestreo, obteniendo de ese modo el número medio de flores de cada linaje abiertas en cada población o rodal durante cada una de las horas del día. A partir de estos

datos promediados de los censos, para cada población o rodal se calculó el valor de aislamiento por asincronía en la antesis diaria (**$RI_{antesis}$**) usando la misma ecuación que en el apartado anterior, esto es la ecuación RI_{4S2} propuesta por Sobel and Chen (2014):

$$\mathbf{RI}_{\mathbf{antesis}} = \mathbf{1} - \mathbf{2} \times \left[\frac{\frac{\sum_i \left(\frac{A_i}{A_{total}} \times \frac{B_i}{A_i + B_i} \right)}{B_{total} / (A_{total} + B_{total})}}{\frac{\sum_i \left(\frac{A_i}{A_{total}} \times \frac{B_i}{A_i + B_i} \right)}{B_{total} / (A_{total} + B_{total})} + \frac{\sum_i \left(\frac{A_i}{A_{total}} \times \frac{A_i}{A_i + B_i} \right)}{A_{total} / (A_{total} + B_{total})}} \right]$$

En este caso, los distintos términos de esta ecuación representan lo siguiente:

- "**A_i / A_{total}**" representa la razón entre el número medio de flores del linaje A (para el que estamos calculando el valor de la barrera) en antesis durante la hora i y el número medio de horas de antesis acumuladas a lo largo de un día por 100 flores de ese linaje.

- "**$A_i / (A_i + B_i)$**" representa la razón entre el número medio de flores del linaje objeto del cálculo (A) en antesis durante la hora i y el número medio de flores de ambos linajes en conjunto en antesis durante esa misma hora.

- "**$B_i / (A_i + B_i)$**" representa la razón entre el número medio de flores del otro linaje (B) en antesis durante la hora i y el número medio de flores de ambos linajes en conjunto en antesis durante esa misma hora.

- "**$A_{total} / (A_{total} + B_{total})$**" o "**$B_{total} / (A_{total} + B_{total})$**" representa la razón entre la media de horas de antesis acumuladas a lo largo de un día por 100 flores del linaje A o por 100 flores del linaje B, respectivamente, y el número medio de horas de antesis acumuladas a lo largo de un día por 200 flores, 100 de cada linaje.

De este modo, se calculó el valor de esta barrera para cada linaje en cada una de las poblaciones o rodales estudiados, y después se promediaron los valores de las mismas para obtener un valor medio de aislamiento por asincronía en la antesis diaria (**$RI_{antesis}$**) para cada linaje.

Aislamiento mediado por polinizadores

Para valorar el flujo polínico entre y dentro de linajes mediado por los polinizadores, se establecieron rodales artificiales con diferentes proporciones de cada color floral en tres poblaciones naturales de *L. arvensis* (Sevilla, Dos Hermanas y Santiponce; Sevilla; SW España). Se consideraron tres tipos de rodales: "balanceado" con igual proporción de flores de ambos colores, "sesgado al azul" con un 80% de flores azules y 20% de flores rojas y "sesgado al rojo" con un 20% de flores azules y un 80% de

rojas. Cada rodal estaba compuesto por 12-20 plantas con un total de 100 flores de ambos colores entremezcladas. En cada población se establecieron entre uno y cinco rodales de cada tipo, salvo en la población "Sevilla" donde no se colocaron rodales sesgados al rojo, totalizando siete rodales balanceados, ocho sesgados al azul y cuatro sesgados al rojo. En cada rodal se efectuó un número variable de censos de 15 minutos, siempre entre las 9 y las 14 horas de un día soleado, en los que se registró la secuencia de flores visitadas, considerando su color, por cada polinizador observado en el rodal. En la población de Sevilla, donde la asistencia de los polinizadores fue relativamente baja, se realizaron un total de 10 y 8h de censos en los rodales sesgados al azul y balanceados, respectivamente. En Santiponce, la asistencia de los polinizadores fue más elevada y se realizaron 5, 7 y 8h de censos en los rodales sesgados al azul, balanceados y sesgados al rojo, respectivamente. En la población de Dos Hermanas, se realizaron 8h de censos en cada tipo de rodal. A partir de las secuencias de flores visitadas registradas en cada rodal se cuantificó el número de transiciones entre flores del mismo color, diferenciando ambos colores (de azul a azul, A→A, o de rojo a rojo, R→R), y entre colores distintos, diferenciando el sentido de la transición, de azul a rojo (A→R) o de rojo a azul (R→A). El número de transiciones totales registradas en cada rodal osciló entre 68 y 293.

Con los datos de transiciones registradas en cada rodal se calculó el aislamiento mediado por polinizadores para cada linaje actuando como padre y como madre. Para ello aplicamos la ecuación RI_{4S4} propuesta por Sobel and Chen (2014) que incorpora términos de valores esperados para acomodarse a diferencias de abundancia entre linajes. Dicha ecuación se utilizó tal como la aplican Sobel & Streisfeld (2015) al cálculo del aislamiento mediado por polinizadores de manera específica para cada linaje y sexo; por ejemplo, para calcular el aislamiento del linaje azul cuando actúa como madre aplicamos la siguiente expresión:

$$\mathbf{RI}_{\mathbf{polinizadores}} = \mathbf{1} - \mathbf{2} \times \left[\frac{\frac{\mathbf{Observadas\ (R \rightarrow A)}}{\mathbf{Esperadas\ (R \rightarrow A)}}}{\frac{\mathbf{Observadas\ (R \rightarrow A)}}{\mathbf{Esperadas\ (R \rightarrow A)}} + \frac{\mathbf{Observadas\ (A \rightarrow A)}}{\mathbf{Esperadas\ (A \rightarrow A)}}} \right]$$

Los términos **Observadas (R→A)** y **Observadas (A→A)** representan el número de transiciones inter-linaje e intra-linaje, respectivamente, registradas en el rodal para el linaje azul actuando como madre. Los valores esperados para cada tipo de transición en ese rodal se calculan a partir de las frecuencias de los linajes implicados y del número total de transiciones registradas en el rodal; esto es, **Esperadas (R→A) =** Frecuencia (R) x Frecuencia (A) x Total y **Esperadas (A→A)** = Frecuencia (A) x Frecuencia (A) x Total.

En cada rodal se calculó **RI** $_{\textbf{polinizadores}}$ separadamente para cada linaje cuando éste actúa como padre y cuando actúa como madre; luego se promediaron los valores de **RI** $_{\textbf{polinizadores}}$ correspondientes a cada tipo de rodal (balanceado, sesgado al azul y sesgado al rojo); y por último se promediaron los valores de los tres tipos de rodal para obtener un valor medio de **RI** $_{\textbf{polinizadores}}$ para cada combinación de linaje y género.

Precedencia polínica

Se realizaron polinizaciones controladas para determinar si existe precedencia polínica, esto es si cuando el polen del propio linaje y el del linaje alternativo coinciden en el estigma, uno de los dos (normalmente el propio) tiene ventaja para alcanzar la fecundación de los óvulos (ver Howard 1999). Para ello usamos 27 plantas de flores azules y 32 de flores rojas cultivadas en invernadero. Una vez alcanzada la floración, se emascularon las flores y en cada planta se realizaron tres tipos de tratamientos de polinización. El primer tratamiento (1) consistió en depositar en los estigmas una mezcla 50:50 de polen de ambos linajes. En el segundo tratamiento (2) se depositó primero polen del linaje alternativo y, transcurridas tres horas, se depositó polen del propio linaje sobre los estigmas de las mismas flores. En el tercer tratamiento (3) se depositó en primer lugar polen del propio linaje y, transcurridas tres horas, polen del linaje alternativo. Estos tratamientos tratan de simular situaciones naturales: el tratamiento 1 imita la deposición de polen simultanea de ambos linajes producida por polinizadores que visitan flores de los dos colores indistintamente; mientras que los tratamientos 2 y 3 simulan la polinización con polen del mismo linaje antes (3) o después (2) de la polinización con polen del linaje diferente. Para todos los tratamientos, se usaron cargas de polen de dos plantas distintas por linaje. La mezcla de polen se obtuvo utilizando anteras completas cuyo polen se recogió en tubos de microcentrífuga limpios, el primer día de antesis, antes de la dehiscencia. Para el tratamiento 1, las anteras de flores de distinto color en una proporción 1:1 se mezclaron mediante sonicación y centrifugación directamente en los tubos en los que se recogían. Para cada tratamiento de polinización, el polen se aplicó en los estigmas en cantidades saturantes en el primer día de antesis, usando un pincel pequeño. Todos los frutos obtenidos de cada tratamiento se cosecharon una vez que las semillas estaban maduras. Para estimar la proporción de descendencia híbrida producida después de cada uno de los tratamientos de polinización, las 1946 semillas obtenidas se sembraron en macetas y las plantas se cultivaron hasta que llegaron a florecer anotándose el color de las flores. Solo 1064 plantas llegaron a florecer y se pudo anotar el color de las flores: 225 del tratamiento 1, 438 del 2 y 401 del 3. Si el color de las flores de la F_1 coincidía con el del progenitor materno, la descendencia se consideró pura (intra-linaje), es decir el progenitor paterno era del mismo linaje que el materno; cuando el color de la F_1 era salmón se consideró descendencia híbrida (inter-

linaje; ver capítulo 2), es decir el progenitor paterno era del linaje alternativo al materno.

Para estimar la fuerza del aislamiento reproductivo originado por la precedencia del polen (**RI** $_{\textbf{precedencia}}$) solo se utilizó la descendencia obtenida con el tratamiento 1 en el que el polen de ambos linajes competía en similar proporción y simultáneamente durante la polinización. En esta situación, si no existe precedencia polínica alguna, se espera que cada planta materna produzca igual proporción de descendencia intra-linaje e inter-linaje. Para estimar el valor de **RI** $_{\textbf{precedencia}}$ se utilizó la ecuación RI_{4S4} de Sobel and Chen (2014) que incorpora términos de valores esperados para acomodarse a diferencias de abundancia entre linajes, en este caso diferencias en número de flores usadas y posibles diferencias en número de óvulos por flor:

$$\mathbf{RI}_{\mathbf{precedencia}} = \mathbf{1} - \mathbf{2} \times \left[\frac{\frac{\mathbf{H\ observados}}{\mathbf{H\ esperados}}}{\frac{\mathbf{H\ observados}}{\mathbf{H\ esperados}} + \frac{\mathbf{C\ observados}}{\mathbf{C\ esperados}}} \right],$$

donde **H** es el número de descendientes inter-linaje y **C** el de descendientes intra-linaje. El valor de **RI** $_{\textbf{precedencia}}$ se calculó para cada linaje y género por separado; para el cálculo del valor de esta barrera para un linaje actuando como padre se consideró que sus descendientes intra-linaje son los del mismo color producidos por las plantas maternas de ese linaje y sus descendientes inter-linaje son los híbridos producidos por las plantas maternas del linaje alternativo.

Barreras postcigóticas

Para evaluar la presencia de barreras postcigóticas entre los linajes, se compararon los valores de diferentes componentes del fitness de la descendencia obtenida a partir de cruces manuales inter-linaje e intra-linaje. Los componentes de fitness medidos a lo largo del ciclo de vida fueron: formación de semillas por fruto, porcentaje de germinación de las semillas, supervivencia de las plántulas hasta la reproducción, producción de polen y de óvulos de esa F_1 y producción de semillas por fruto de dicha F_1.

Para ello se realizaron polinizaciones manuales en el invernadero en flores emasculadas de 22 plantas de flores azules (A) y 41 de flores rojas (R). Se realizaron un total de 248 cruces inter-linaje (128 AxR, madre azul-padre rojo; 120 RxA, madre roja-padre azul) y 200 cruces intra-linaje (66 AxA; 144 RxR). Cuando los frutos resultantes estuvieron maduros, se recolectaron separadamente los de cada tipo de cruce y se contó el número de semillas de cada fruto. Las semillas obtenidas de estos frutos (1920) se sembraron en semilleros con turba. La procedencia de estas semillas

según el tipo de cruce fue la siguiente: 192 AxR, 192 RxA, 786 AxA y 786 RxR. Los semilleros se dispusieron en dos poblaciones naturales de *L. arvensis* próximas a la ciudad de Sevilla. Los semilleros recibieron únicamente el agua de la lluvia y durante los cuatro primeros meses se censaron dos veces por semana a fin de controlar la germinación y supervivencia de las plántulas hasta la floración. Posteriormente, los censos se realizaron una vez por semana hasta que se completó la maduración de los frutos producidos por polinización libre. En una submuestra de estas plantas (5-13 plantas por tipo de cruce, 37 plantas en total) se cuantificó la producción de polen y óvulos para lo que se recolectaron uno o dos botones florales por planta. En cada botón floral, se extrajeron mecánicamente los granos de polen de las cinco anteras y se estimó su número mediante un contador de partículas (Multisizer 3 Coulter Counter. Beckman coulter. California). Para los mismos botones florales se contabilizó también la producción de óvulos. Una vez los frutos de polinización libre estuvieron completamente maduros, se recolectaron 1-3 frutos por planta (n= 419 frutos. 99 RxA, 82 AxR, 105 AxA y 133 RxR) y se contaron sus semillas.

Para valorar la posible pérdida de viabilidad en sucesivas generaciones se cuantificó la producción de semillas de la F_2. En las plantas F_1 cultivadas en invernadero se realizaron polinizaciones cruzadas manuales entre plantas del mismo fenotipo (AxA, RxR y SxS), entre fenotipos parentales (AxR) y retrocruces (AxS y RxS); además se permitió que todas las plantas formaran semillas por autopolinización. Las semillas formadas se sembraron en placas de Petri, diferenciando los 6 tipos de cruces (AxA, RxR, SxS, AxR, AxS y RxS), y se expusieron, en germinadoras, a un ciclo de 14h luz/10h oscuridad con 22ºC y 15ºC, respectivamente. Tras la germinación, las plántulas se traspasaron a macetas individuales y se cultivaron hasta la fructificación. Para cada planta se contabilizó el número de flores transformadas y no transformadas en frutos, con lo que se estableció el porcentaje de fructificación por planta (n=195). Además, para algunas de esas plantas se recolectaron 2 cápsulas (n=98 plantas), formadas por autopolinización, y se cuantificó el número medio de semillas por fruto de cada planta. Por otro lado, se realizaron polinizaciones cruzadas manuales entre plantas de la F_2 (n=60) para descartar posibles efectos de la autopolinización en la fructificación y producción de semillas.

Se calculó el valor del aislamiento reproductivo para cada uno de los componentes del fitness estudiados en la F_1: cuajado de semillas F_1 por los parentales, germinación, supervivencia, producción de polen, producción de óvulos y cuajado de semillas F_2 por las plantas F_1. Nos referiremos a estas barreras individuales como $RI_{cuajado1}$, $RI_{germinación}$, $RI_{supervivencia}$, RI_{polen}, $RI_{óvulos}$ y $RI_{cuajado2}$, respectivamente. Estos cálculos se hicieron siguiendo a Sobel and Chen (2014), mediante la siguiente ecuación:

$$RI_{\text{postcigóticos}} = 1 - 2 \times \left(\frac{H}{H + C}\right)$$

En cada caso, **H** es el valor del componente del fitness para la descendencia de cruces inter-linaje y **C** es el valor del mismo para la descendencia de cruces intra-linaje. El valor de cada barrera se calculó para cada combinación de linaje y género.

Aislamiento reproductivo acumulado

Se calculó además el aislamiento reproductivo total acumulado por todas las barreras pre- y post-cigóticas estudiadas actuando secuencialmente. Para ello se utilizó directamente la hoja Excel proporcionada en Sobel and Chen (2014) como material suplementario (evo12362-sup-0003-SuppMat.xls); esta hoja está preparada para introducir los valores de las distintas barreras individuales ordenadas según su secuencia temporal y va calculando directamente el valor total acumulado, así como la contribución relativa de cada barrera al aislamiento total. Esta hoja se utilizó separadamente para cada combinación de linaje y género, así como para cada linaje. Para estos cálculos no se utilizó la barrera $RI_{\text{óvulos}}$ porque esa barrera sería redundante con RI_{cuajado2}, ya que la segunda depende de la primera. El aislamiento total y la contribución relativa de cada barrera se estimó utilizando tanto el conjunto de las 10 barreras consideradas, como, sin tener en cuenta la barrera geográfica, tal y como proponen algunos trabajos previos (Runquist et al. 2014, Sobel & Chen 2014; Sobel & Streisfeld, 2015), los cuales defienden la idea de estudiar las barreras solo el área en simpatría, puesto que es el único área donde pueden darse eventos de flujo génico cruzado entre las entidades estudias.

Análisis estadísticos

Los datos se analizaron utilizando modelos lineales generalizados (GLM) con diferentes funciones de enlace y distribuciones de errores, según el tipo de respuesta variable modelada. Las diferencias en el periodo de floración entre linajes se analizaron mediante un GLM con distribución de normal con función de enlace identidad, se usó la variable días de floración como dependiente y color como factor fijo. Además, se comparó el pico de floración de cada linaje entre poblaciones mediante un GLM con distribución multinomial y función de enlace logit acumulado, la variable dependiente fue el mes, en el cual se dio el pico de floración, y como factores fijos se establecieron las poblaciones, el color floral y la interacción. Las diferencias en el número de flores de cada color abiertas a lo largo del día se analizaron mediante un GLM con distribución de probabilidad Poisson y función de enlace logarítmica. Se usó el número de flores abiertas como variable dependiente, siendo el color y las horas del día los factores fijos.

Para evaluar si los polinizadores realizaban transiciones de igual frecuencia entre las flores del mismo o distinto color, los datos obtenidos se analizaron mediante una distribución binomial usando como variable dependiente el número de transiciones por censo y el tipo de transición (A→A; A→R; R→A; R→R), el color floral y su interacción se incorporaron como factores fijos.

Así mismo, la importancia de la precedencia del polen en la formación de semillas híbridas se analizó mediante un GLM con distribución de probabilidad normal y función de enlace identidad, con la tasa de descendientes hibridas como variable dependiente y como factores fijos, el tratamiento de polinización (1, 2 o 3), color floral de la planta materna (azul o rojo) y su interacción.

Para comparar las medidas de fitness se usó la distribución normal de errores con función de enlace identidad en la producción de polen/antera, óvulos/ovario y semillas por fruto (tanto parental como de la F_1 y F_2), y la distribución Poisson con función de enlace logarítmica en la tasa de germinación y de supervivencia. En todos los casos el rasgo medido de fitness se seleccionó como variable dependiente y como factores fijos el tipo de cruce (AxA; AxR; RxA; RxR), el color floral, tipo de progenie (parental o hibrida) y la interacción entre tipo de progenie y color.

Todos estos análisis se realizaron utilizando el módulo GLM de SPSS (IBM SPSS Statistic 25, 2017, EE. UU.) Con una prueba de tipo III. Cuando los GLM mostraron diferencias significativas, las medias de cada tratamiento se compararon mediante pruebas t basadas en los errores estándar calculados a partir de los modelos específicos.

RESULTADOS

Barreras precigóticas

En base al índice de jackknife en el ENM de *L. arvensis*, se seleccionaron 8 variables ambientales para estimar el área de distribución de cada linaje: 4 coincidentes y 4 diferentes para cada linaje. Las 4 variables bioclimáticas compartidas fueron BIO5, BIO10, BIO13, y BIO17 (Tabla 1). Las diferentes estuvieron relacionadas con la temperatura (BIO2 y BIO8, en el rojo; BIO3 y BIO7 en el azul) y con la precipitación (BIO10 y BIO18, en el rojo; BIO16 y BIO19 en el azul) (Tabla 1). Todas las proyecciones realizadas mostraron excelentes tasas de éxito predictivo, con valores AUC superiores a 0,9. El ENM para las condiciones ambientales actuales fue altamente concordante con la distribución actual de la especie y cada linaje. Las áreas potenciales de contacto se predijeron en menos de la mitad (37,14%) de la distribución en el Mediterráneo y Europa. La probabilidad de co-ocurrencia de ambos linajes fue

superior en la Península Ibérica y Cuenca Mediterránea. El linaje rojo presentó un área proyectada más amplia que el linaje azul, especialmente en el centro de Europa (Fig. 1). El linaje azul mostró una ligera incidencia superior en el norte de África que el linaje rojo, pero su área fue en general más pequeña y altamente asociada a la Cuenca Mediterránea. Los resultados predijeron que en el 62,86% del área total natural de la especie solo ocurre uno de los linajes. Respecto al área del linaje azul, se predijo un 74,54% de la misma en simpatría con el linaje rojo, siendo un 42,53% el área del este último en simpatría con el linaje azul (Fig. 1).

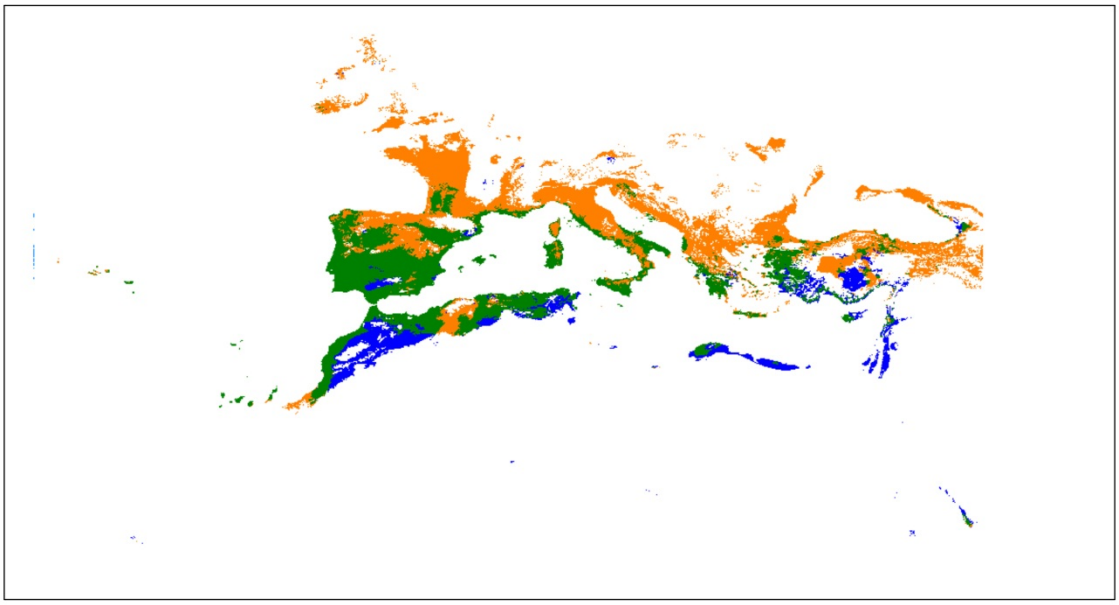

Figura 1. Área de distribución natural de *Lysimachia arvensis* obtenida a través de Modelado de nicho ambiental (ENM). Se representan el área ocupada exclusivamente por el linaje rojo (Naranja), el ocupada solo por el linaje azul (Azul), y el área de coexistencia (Verde).

La fenología de la floración se mostró muy variable entre las poblaciones estudiadas y osciló entre marzo y julio (Fig. 2). El linaje azul de *L. arvensis* presentó un período medio de floración significativamente superior (75 ± 3,6 días) que en el rojo (56,64 ± 11,5 días; Wald chi^2 = 12,584; 1gl; p=0,000), aunque en la mayoría de las poblaciones el pico de floración de ambos linajes fue altamente coincidente (interacción Wald chi^2 = 0,000; 21gl; p=1,000). En el periodo de floración seis de las once poblaciones mostraron un ligero desacople entre el periodo de floración del linaje azul, más precoz, y el del linaje rojo, más tardío. Este desfase fue especialmente importante en una población de Zahara de la Sierra (ESP_CA_ZS1), otra de Aracena (ESP_H_AR4) y otra de Sevilla (ESP_SE_Al) donde el pico máximo de floración del linaje rojo coincidió con el declive en la floración del linaje azul (Fig. 2). Sin embargo, se observaron otras poblaciones donde la floración de ambos linajes fue bastante sincrónica (ej. ESP_H_Hin; Fig. 2).

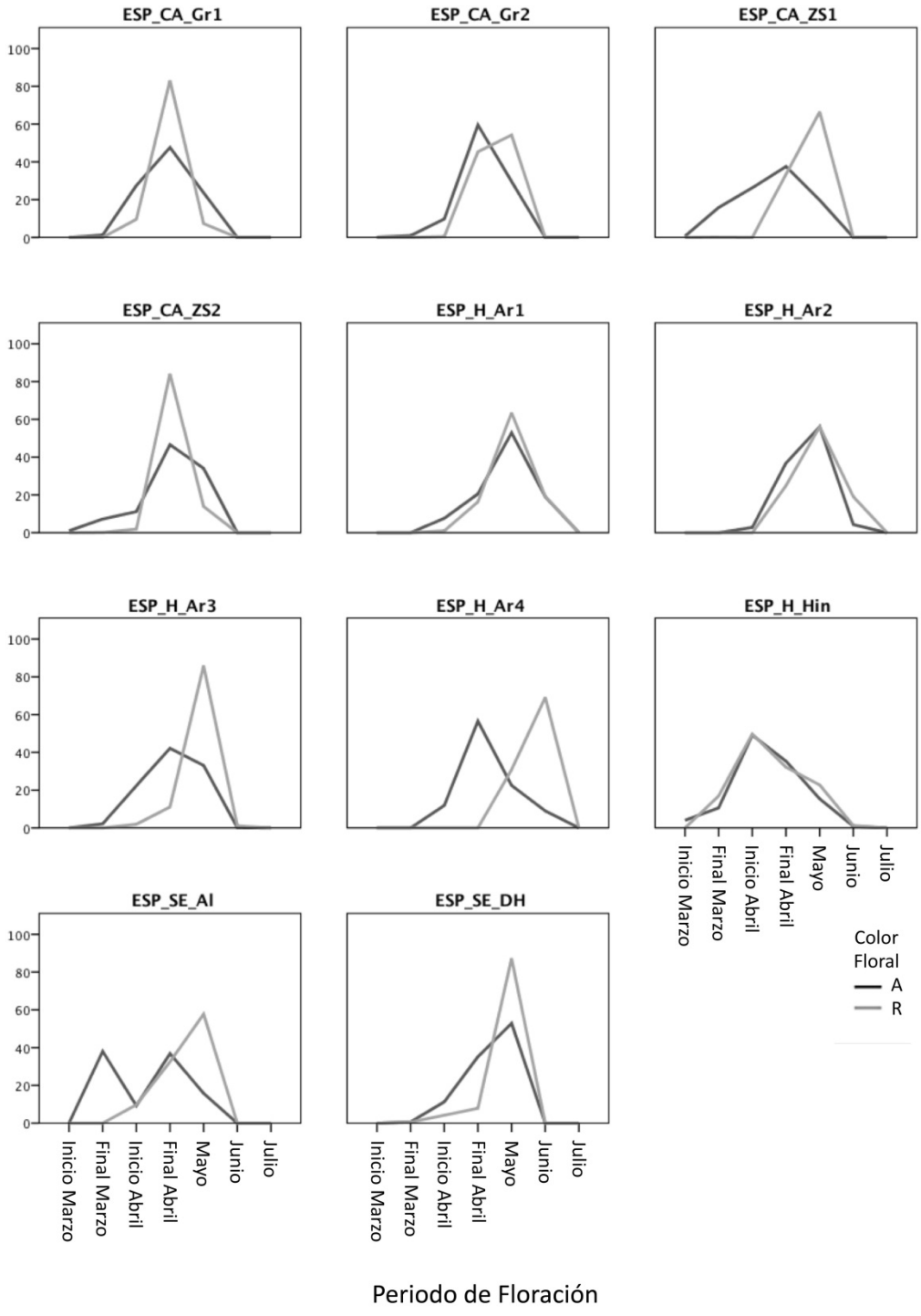

Figura 2. Fenología floral estacional en once poblaciones mixtas naturales de *Lysimachia arvensis* (Tabla 2). Se representa el porcentaje de flores abiertas de cada linaje a lo largo del periodo completo de floración.

La fenología de la floración diaria difirió entre linajes, ya que el azul abrió sus flores antes y las cerró después que el rojo. Como consecuencia, las flores azules estuvieron abiertas una media de 10,25h al día frente a las 8,57h de las flores rojas (Fig. 3). La interacción en la frecuencia de flores abiertas por momento del día fue

significativamente diferente entre ambos linajes (Wald chi^2 = 9576,638, 21 gl, p< 0,0001), debido a la proporción inferior de flores rojas abiertas en las primeras cuatro horas y tres últimas horas del día, respecto de las flores azules.

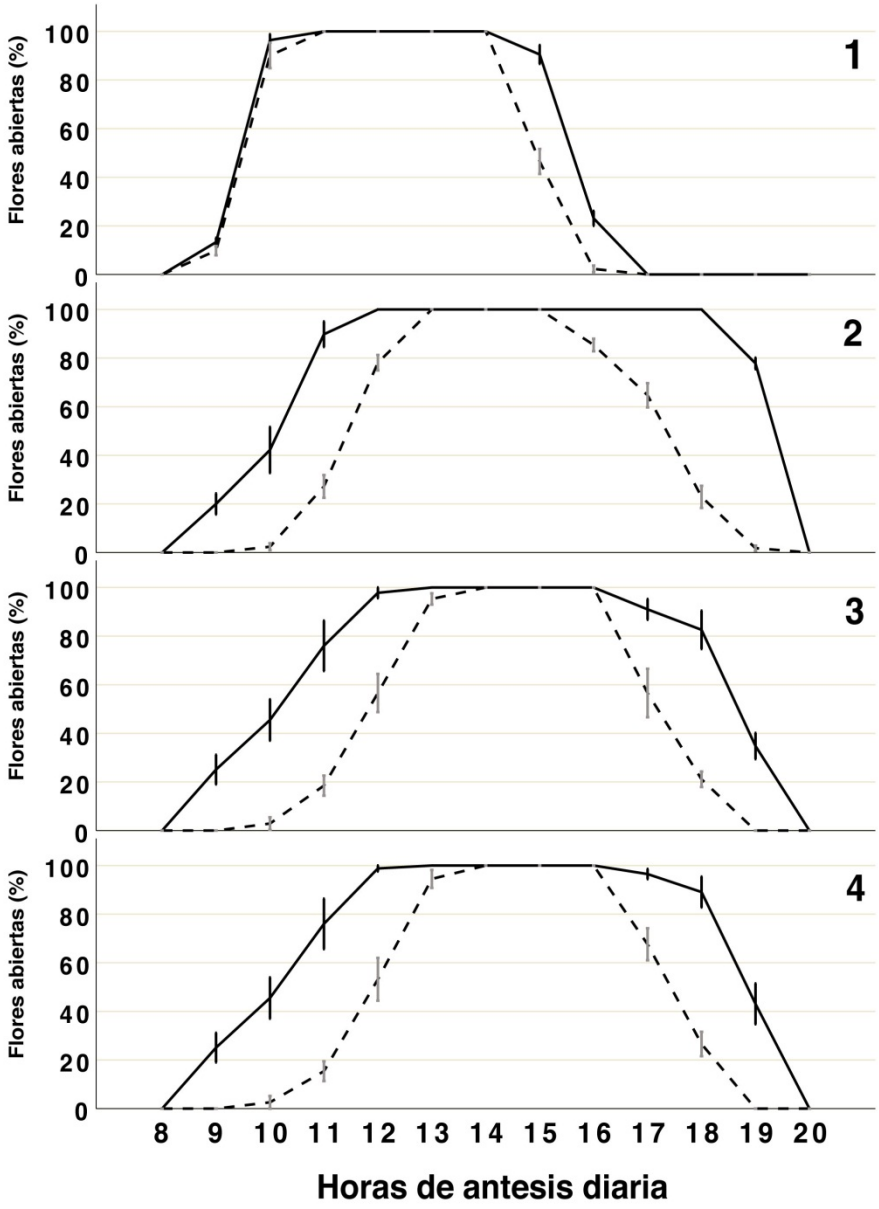

Figura 3. Fenología floral diaria en cuatro poblaciones mixtas naturales de *Lysimachia arvensis*. Se representa el porcentaje de flores abiertas de cada linaje a lo largo del día. Los censos se distribuyeron a lo largo del periodo de floración de la especie. Poblaciones: 1: Lausanne (Suiza); 2: Santiponce (SE); 3: Bellavista (SE); 4: Dos Hermanas (SE) (España).

Las diferencias en los patrones de apertura de ambos linajes hicieron que el periodo de antesis del rojo estuviera siempre comprendido dentro del periodo del azul.

Con respecto al aislamiento por polinizadores, en todos los censos se observó que las abejas solitarias de pequeño tamaño fueron las principales visitantes de *L. arvensis*,

aunque las flores también recibieron visitas puntuales de sírfidos. De las 3012 transiciones realizadas por los polinizadores, 2257 fueron intralinaje (1502 entre azules y 755 entre rojas) y 755 entre linajes (393 A→R y 362 R→A). En general en las tres poblaciones el patrón fue muy parecido para los tres tipos de rodales con una mayor cantidad de transiciones dentro del linaje azul que dentro del rojo (Fig. 4), siendo estas diferencias significativas (Wald chi^2 = 15,292, 1 gl, p<0,0001). Las transiciones entre linajes de distinto color, no mostraron en general ningún patrón, (Wald chi^2 = 0,231, 1 gl, p<0,631), es decir, el numero de transiciones fue similar en ambos sentidos salvo la población de Dos Hermanas donde, tanto el rodal 80A:20R como 50A:50R mostraron significativamente más transiciones A→R que R→A (Wald chi^2 = 4,297, 1 gl, p<0,038) (Fig. 4).

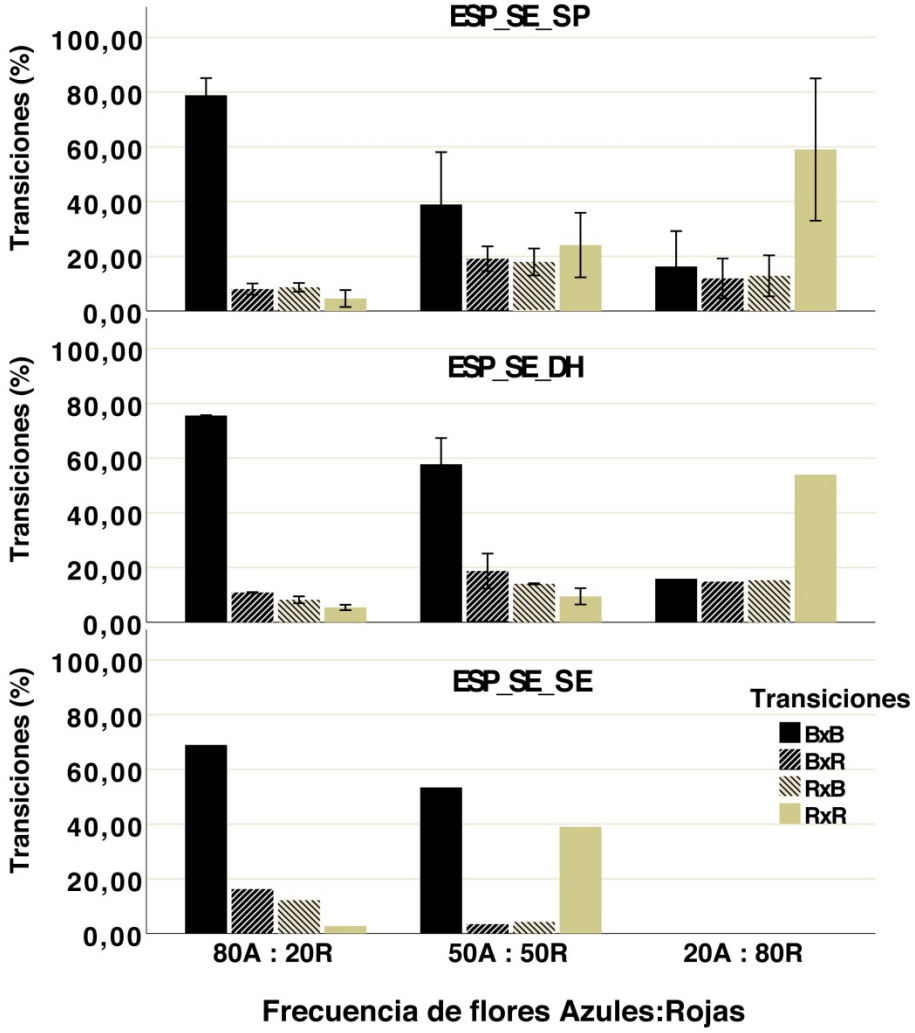

Figura 4. Porcentaje de transiciones entre flores del mismo o distinto color realizadas por los polinizadores en censos de 15 minutos en stands experimentales con diferentes proporciones de cada color floral (80A:20R, 50A:50R y 20A:80R) situados en tres poblaciones naturales en el sur de la Península Ibérica. Poblaciones: ESP_SE_SP: Santiponce (Sevilla), ESP_SE_DH: Dos Hermanas (Sevilla), ESP_SE_SE: Parque infanta Elena (Sevilla) (España). Se muestra la media y la desviación estándar.

En los experimentos para conocer la importancia de la precedencia polínica en la producción de plantas hijas, nuestros resultados mostraron que, para los tres tratamientos utilizados, no existía correlación entre la producción de plantas "híbridas" esperadas y observadas en el linaje azul (R = 0,142; p=0,279). Sin embargo, en el linaje rojo esta correlación si fue significativamente positiva (R = 0,676; p=0,007). En cuanto a los tratamientos, se observó una correlación negativa, aunque no significativa (R=-0,258; p=0,256), para el tratamiento 1, correlación significativamente positiva para el tratamiento 2 (R=0,887; p=0,000) y correlación positiva no significativa en el tratamiento 3 (R=0,576; p=0,116). En las madres azules la producción de descendencia híbrida fue menor de la esperada para los tratamientos 1 (15,57%) y 2 (11,35%), por el contrario, en las madres rojas la producción de descendencia hibrida fue mayor de la esperada en los tratamientos 1 (73, 88%) y 3 (22,49%). Para el conjunto de los datos, la descendencia producida por polen heteromórfico fue significativamente inferior (Wald chi^2 = 162,284; 1 gl; p=0,000) (Fig. 5). Fue destacable la frecuencia de descendencia híbrida obtenida en el tratamiento 1, deposición simultanea de pólenes, donde el polen del linaje azul fue más exitoso de lo esperado indistintamente del fenotipo materno (84,43%para madres azules y 73,88% para madres rojas. En general el polen procedente del linaje rojo fue significativamente menos exitoso de lo esperado en todos los casos (Wald chi^2 = 230,128; 11 gl; p=0,000).

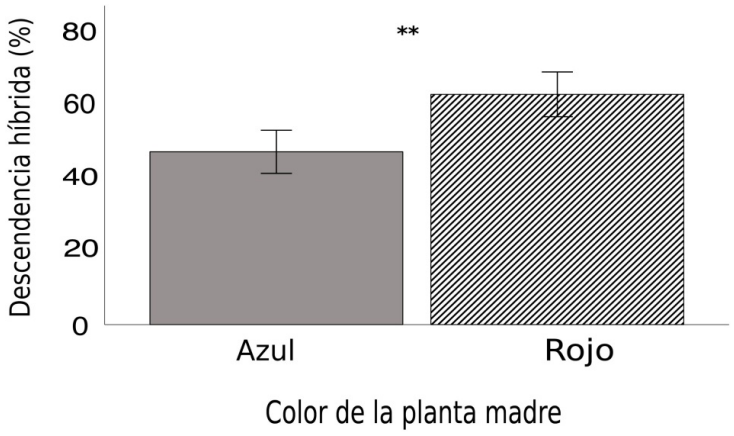

Figura 5. Proporción de descendencia intralinaje e interlinaje obtenida tras polinización de cada linaje de *L. arvensis* con una mezcla equilibrada de polen de ambos linajes. Se considero descendencia intralinaje cuando el color floral de la F1 coincidía con de la planta madre y descendencia interlinaje o híbrida cuando la F_1 manifestó el fenotipo "Salmón". ** p < 0,05

Barreras postcigóticas en la F_1

La producción de semillas tras polinización con polen del mismo o diferente linaje solo mostró relación con el linaje materno (Tabla 3). En general las madres del linaje rojo

produjeron 1,5 veces más semillas que las del azul, independientemente de la procedencia del polen (número medio de semillas por tipo de cruce: AxA = 12,24, AxR = 14,68, RxA = 19,97 y RxR = 19,81; Fig. 6A).

Tabla 3. Resumen de los resultados de GLM comparando diferentes rasgos de fitness en la F1 y F2 de *Lysimachia arvensis* en función del Tipo de descendencia (parental/híbrida), Tipo cruce (AxA, AxR, RxA y RxR), color floral (azul/rojo/salmón) y sus interacciones. En **negrita** se muestran los valores significativos.

Variable dependiente	**Efectos**	**Wald chi2**	**Gl**	***P***
Produccion de semillas (Parental)	Cruce	53,662	3	**0,000**
	Color	31,788	2	**0,000**
Tasa Germinación	Descendencia	0,303	1	0,582
	Cruce	31,049	3	**0,000**
	Color	29,309	2	**0,000**
	D x C	0,215	1	0,643
Tasa Supervivencia	Descendencia	0,288	1	0,591
	Cruce	0,124	3	0,725
	Color	4,523	2	0,104
	D x C	0,315	1	0,575
Producción polen	Descendencia	0,204	1	0,651
	Cruce	35,551	3	**0,000**
	Color	1,780	2	0,411
	D x C	1,940	1	0,164
Producción óvulos	Descendencia	2,523	1	0,112
	Cruce	10,267	3	**0,016**
	Color	2,523	2	**0,037**
	D x C	8,703	1	**0,003**
Producción semilla (F_1)	Descendencia	11,171	1	**0,001**
	Cruce	30,788	3	**0,000**
	Color	29,389	2	**0,000**
	D x C	269,546	1	**0,000**
Fructificación (F_2)	Descendencia	113,182	1	**0,000**
	Cruce	135,070	2	**0,000**
	Color	11,170	2	**0,004**
	D x C	0,000	1	0,996
Producción Semillas (F_2)	Descendencia	35,833	1	**0,000**
	Cruce	40,330	2	**0,000**
	Color	0,340	2	0,844
	D x C	0,034	1	0,853

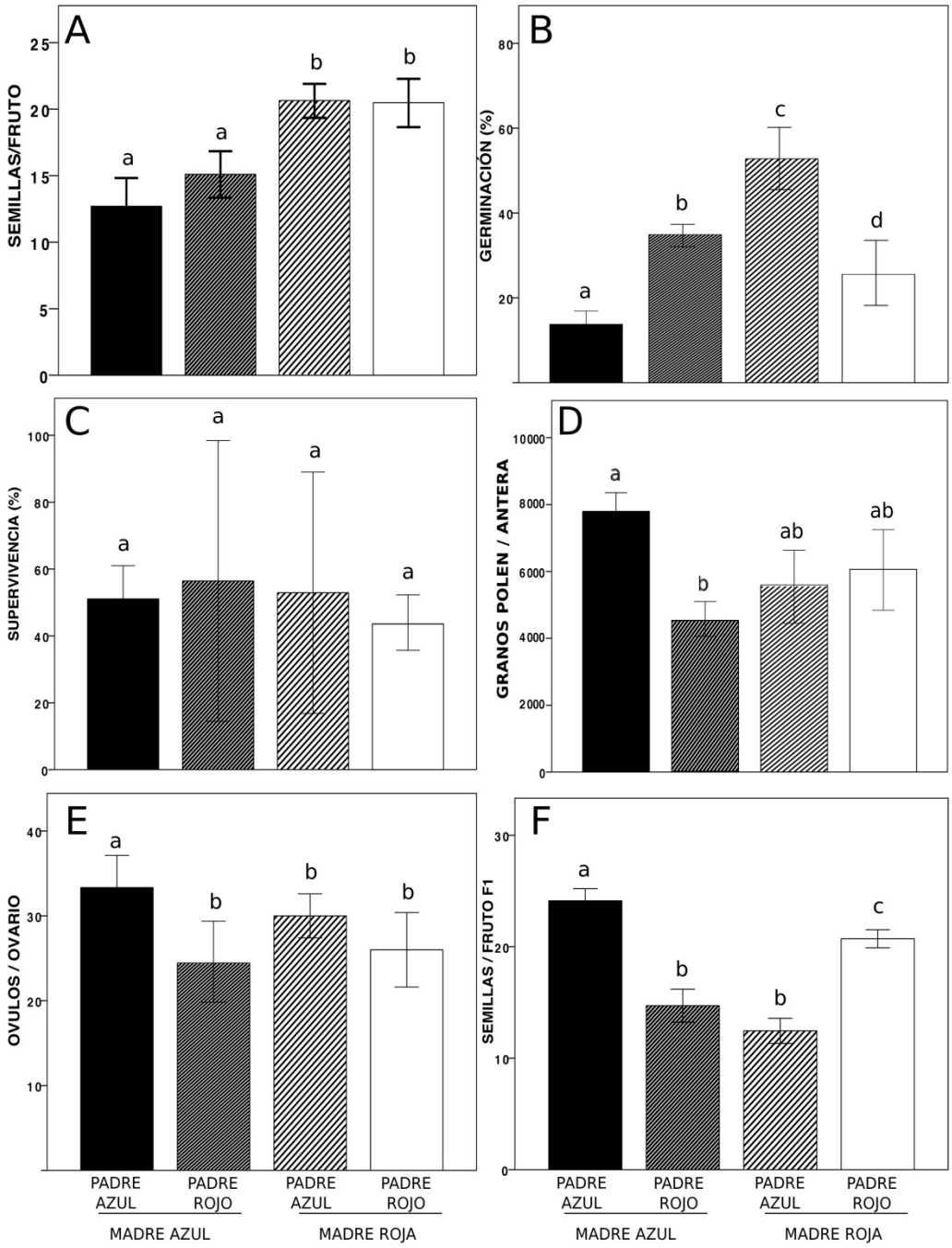

Figura 6. Diferencias para 6 rasgos del fitness de la de la progenie F_1 obtenida a partir de cruces inter- e intralinajes en *L. arvensis*. En cada rasgo, diferentes letras indican diferencias significativas ($p<0,05$). A: Número medio de semillas por fruto de los cruces entre parentales; B: Tasa media de germinación; C: Tasa media de supervivencia; D: Número medio de granos de polen por antera; E: Número medio de óvulos por ovario; F: Número medio de semillas por fruto producido por la F_1.

El porcentaje de germinación observado de esas semillas mostró, sorprendentemente, un mayor éxito en las plántulas de origen híbrido (AxR =35,79%; RxA = 5,34%) que en las producidas por cruces entre plantas azules (13,43%) o rojas (25,91%) (Fig. 6B), aunque los cuatro tipos de cruces fueron significativamente distintos entre si (Tabla 3).

El linaje azul mostró 4 veces mayor éxito como padre en madres rojas que en madres azules.

A pesar de las diferencias en germinación, los porcentajes de supervivencia fueron muy similares entre todos los tipos de plántulas (AxA = 51,08%, AxR = 50,48%, RxA = 58,87% y RxR = 48,47%; Fig. 6C), y no se encontraron diferencias significativas entre ellos (Tabla 3).

En las plantas adultas, la producción de polen presentó diferencias significativas (Tabla 3) debidas a la cantidad inferior de polen producida en las plantas originadas del cruce AxR (media 4544) y la significativa elevada cantidad producida en las plantas originadas del cruce AxA (media 7795). Los otros dos cruces RxA y RxR dieron cantidades intermedias y parecidas entre si, 5300 y 5890 granos/antera (Fig. 6D), respectivamente. El número medio de óvulos por flor fue de 33,3, 25, 29 y 26 para los cruces AxA, AxR, RxA y RxR, respectivamente (Fig. 6E). Solo las plantas resultantes del cruce AxA tuvieron una producción de óvulos significativamente mayor que el resto (Tabla 3).

En estas plantas, la producción media de semillas por fruto difirió significativamente entre los cruces interlinaje e intralinaje (Tabla 3). Las mayores cantidades de semillas producidas por fruto se observaron en los cruces dentro del linaje azul (media 24,12), seguido por los del rojo (20,71); los cruces entre linajes produjeron un número significativamente menor de semillas (AxR: 14,71 y RxA: 12,45) (Fig. 6F).

Barreras postcigóticas en la F_2

De las plantas híbridas estudiadas de la F_2, 35 fueron totalmente estériles ya que no produjeron ningún fruto como madre ni como padre (Fig. 7A). La fertilidad del resto de las plantas dependió fuertemente de su origen (Wald chi^2 = 135,070; 2 gl; p = 0,000) (Tabla 3). Cuando uno de los progenitores tenía origen híbrido y el otro no, el porcentaje de fructificación de la progenie fue significativamente inferior al encontrado en plantas obtenidas de cruces intralinaje (64,83% frente al 99,8%). La tasa se fructificación fue aun menor cuando ambos progenitores fueron híbridos (45,38%) (Fig. 7B). Además, los cruces manuales entre plantas de la F_2 mostraron diferencias significativas en la producción de semillas por fruto (Wald chi^2 = 241,957; 2 gl; p=0,000). Los cruces entre híbridos presentaron el menor número medio de semillas por fruto (1,85 $\pm$ 0,525) seguido por los de los retrocruces con número medio de semillas leve pero significativamente superior (3,76 $\pm$ 0,650) y, finalmente, por los de los cruces dentro de linajes puros fueron los que más semillas formaron (14,41 $\pm$ 0,650) (Fig. 7D).

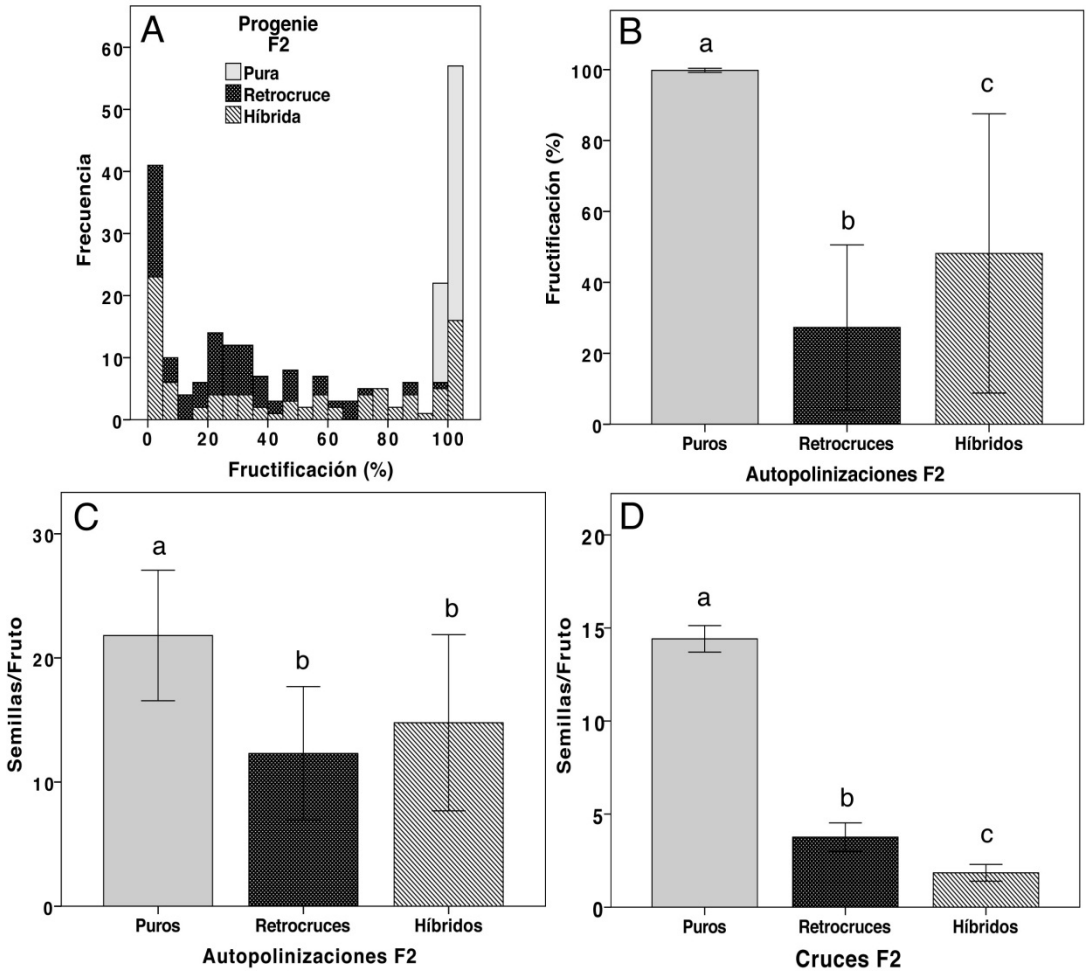

Figura 7. Diferencias en los rasgos de fitness de la progenie F_2 obtenida a partir de cruces manuales entre plantas híbridas entre sí y entre plantas híbridas con plantas puras (retrocruces). Frecuencia de fructificación (A), tasa de fructificación (B) y producción de semillas por autopolinización (C) y polinización cruzada (D). Para cada variable se comparan los cruces **puros** dentro de cada linaje (AxA, RxR), cruces entre **híbridos** (SxS) y **retrocruces** (AxS y RxS). En cada rasgo letras diferentes indican diferencias significativas ($p<0,05$).

Aislamiento reproductivo

Aislamiento precigótico

Para la barrera geográfica, el valor de $RI_{geográfico}$ entre linajes fue de 0,4146. Se observó asimetría en el $RI_{geográfico}$ para cada linaje, siendo algo más de la mitad en el azul (RI_{geo} = 0,2545) y considerablemente superior para el rojo ($RI_{geográfico}$ = 0,5747) (Fig. 8A)

Sin ninguna otra barrera precigótica, en poblaciones mixtas y suponiendo un apareamiento aleatorio, las diferencias fenológicas resultarían en una probabilidad de formación desigual de híbridos que sería igual a las frecuencias en la producción de flores, hipotéticamente. Sin embargo, como el número proporcional de flores abiertas

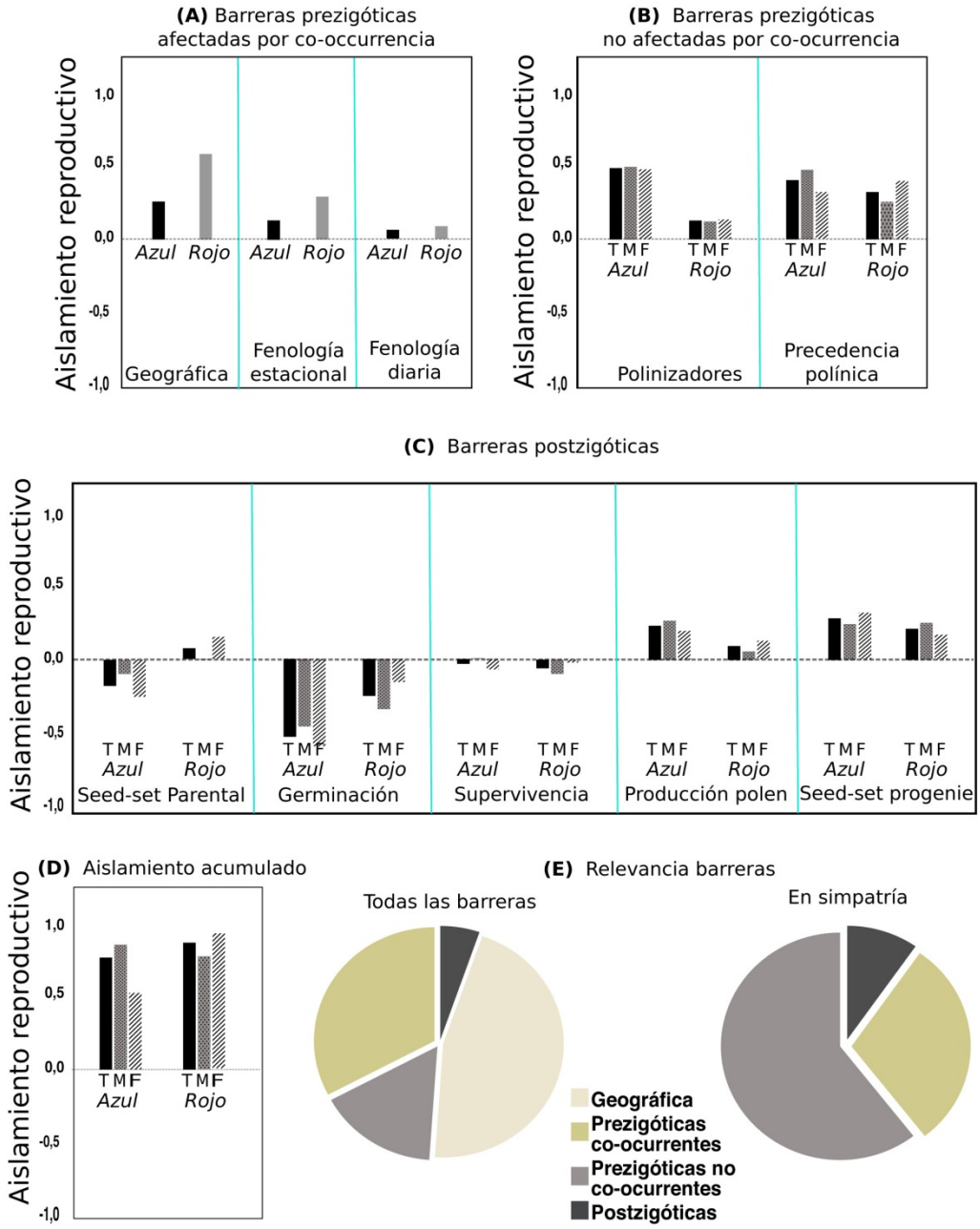

Figura 8. Valores de aislamiento reproductivo para cada una de las barreras estudiadas y su contribución al aislamiento total en *Lysimachia arvensis*. Para cada linaje (Azul o Rojo) se indica el valor total (T) de cada barrera, así como el aislamiento cuando actúa como madre (M) o padre (F).

en sincronía difirió entre los dos linajes, la disponibilidad de polen heteromórfico se redujo y por tanto la probabilidad de formación de híbridos F1 en relación con las expectativas de apareamiento aleatorio. Esto resultó en un $RI_{fenológico}$ de 0,2088, mostrando nuevamente asimetría para ambos linajes, más del doble en el rojo ($RI_{fenológico}$ = 0,2873) que para el azul ($RI_{fenológico}$ = 0,1303) (Fig. 8A). El patrón diario de apertura floral supuso una barrera relativamente pequeña. Dadas las diferencias en el periodo de antesis de cada linaje la disponibilidad de polen heteromórfico fue distinta para cada linaje, siendo inferior para el linaje azul. La magnitud de esta barrera al

aislamiento de *L. arvensis* fue $RI_{antesis}$ = 0,0721, siendo el valor de $RI_{antesis}$ = 0,0589 para el azul y de $RI_{antesis}$ = 0,0852 para el rojo (Fig. 8A).

En general, el comportamiento selectivo de los polinizadores en *L. arvensis* dio lugar a una fuerte barrera para la formación de híbridos F_1 con un valor de $RI_{polinizadores}$ = 0,3024. Esta barrera mostró una marcada asimetría en cada linaje, independientemente que actúen como padre (F) o como madre (M). El linaje azul mostró valores de aislamiento reproductivo ($RI_{polinizadores}$ = 0,4787; [F] $RI_{polinizadores}$ = 0,04703, [M] $RI_{polinizadores}$ = 0,4871) casi cuatro veces superiores al del linaje rojo ($RI_{polinizadores}$ = 0,1260; [F] $RI_{polinizadores}$ = 0,1325, [M] $RI_{polinizadores}$ = 0,1196) (Fig. 8B). La precedencia polínica también constituyó una fuerte barrera de aislamiento que fue para el linaje azul de $RI_{precedencia}$ = 0,3586 ([M] $RI_{precedencia}$ =0,4200; [F] $RI_{precedencia}$ = 0,2973), y para el y rojo de $RI_{precedencia}$ = 0,2951 ([M] $RI_{precedencia}$ =0,2308; [F] $RI_{precedencia}$ = 0,3594) (Fig. 8B).

Aislamiento postcigótico

Dado que el cuajado de semillas mostró muy poca reducción tras los cruces entre linajes, su contribución al aislamiento fue baja en el linaje rojo ($RI_{cuajado1}$ = 0,0723; [M] $RI_{cuajado1}$ = -0,0040; [F] $RI_{cuajado1}$ = 0,1487), e incluso negativa en el linaje azul ($RI_{cuajado1}$ = -0,1653; [M] $RI_{cuajado1}$ = -0,0906; [F] $RI_{cuajado1}$ = -0,2400) (Fig. 8C). La germinación de las semillas híbridas fue superior en las de origen híbrido, por lo que su contribución al aislamiento reproductivo fue siempre negativa, tanto en el linaje azul ($RI_{germinación}$ = -0,5228; [M] $RI_{germinación}$ = -0,4541; [F] $RI_{germinación}$ = 0,5915) como en el linaje rojo ($RI_{germinación}$ = -0,2489; [M] $RI_{germinación}$ = -0,3378; [F] $RI_{germinación}$ = -0,1600) (Fig. 8C). Dada la ausencia de diferencias significativas en la supervivencia entre los distintos tipos de plántulas, la contribución de este rasgo al aislamiento reproductivo fue muy baja, para linaje azul $RI_{supervivencia}$ = -0,0325 ([M] $RI_{supervivencia}$ = 0,0059 [F] $RI_{supervivencia}$ = -0,0709) y rojo $RI_{supervivencia}$ = -0,0585 ([M] $RI_{supervivencia}$ = -0,0968 [F] $RI_{supervivencia}$ = -0,0203) (Fig. 8C).

La amplia diferencia encontrada en el linaje azul en la producción de polen de la F_1 el aislamiento reproductivo fue de RI_{polen} = 0,2293 ([M] RI_{polen} = 0,2635, [F] RI_{polen} = 0,1950). Por el contrario, en el linaje rojo no tuvo gran influencia (RI_{prod_polen} = 0,0932; [M] RI_{polen} = 0,0574, [F] RI_{polen} = 0,1290). En función de los resultados observados en la producción de óvulos, los valores estimados de RI fueron: $RI_{óvulos}$ = 0,1105 ([M] $RI_{óvulos}$ = 0,1428, [F] $RI_{óvulos}$ = 0,0781) para el fenotipo azul y $RI_{óvulos}$ = -0,0131 ([M] $RI_{óvulos}$ = -0,0458, [F] $RI_{óvulos}$ = 0,0196) para el rojo (Fig. 8C). La disminución en el cuajado de semillas de los híbridos originó una barrera tanto para el linaje azul ($RI_{cuajado2}$= 0,2807; [M] $RI_{cuajado2}$= 0,2423, [F] $RI_{cuajado2}$= 0,3191) como para el rojo ($RI_{cuajado2}$= 0,2092; [M] $RI_{cuajado2}$= 0,2491, [F] $RI_{cuajado2}$= 0,1693) (Fig. 8C).

Aislamiento reproductivo acumulado

La asimetría en el aislamiento reproductivo para ambos linajes ha sido patente en todas las barreras estudiadas. En el linaje azul nuestros resultados mostraron un valor de aislamiento reproductivo total superior al 72 %, con gran diferenciación cuando este linaje actuaba como madre (RI_{total} = 0,8324) o como padre (RI_{total} = 0,5603). Por otro lado, para el linaje rojo encontramos un aislamiento superior al 85%, también con asimetría cuando actúa como madre (RI_{total} = 0,7648) y como padre (RI_{total} = 0,9114) (Fig. 8D). En general, las barreras precigóticas no afectadas por la coocurrencia (polinizadores y precedencia polínica), fueron las más relevantes para *L. arvensis*, seguidas de las precigóticas afectadas por la coocurrencia, si no tenemos en consideración el aislamiento reproductivo generado por la distribución geográfica de cada linaje de la especie (Fig. 8E).

DISCUSIÓN

El conjunto de resultados obtenidos en este trabajo demuestra que los dos linajes de *L. arvensis* muestran un alto grado de aislamiento reproductivo total, tanto teniendo en cuenta la distribución natural completa de la especie como considerando únicamente el área donde ambos linajes coexisten. Este aislamiento total se debe a la actuación consecutiva de un alto número de barreras, cada una de ellas con una contribución desigual y frecuentemente asimétrica al aislamiento reproductivo. En conjunto las barreras precigóticas tuvieron una importancia mucho mayor en el aislamiento de los dos linajes que las postcigóticas, una situación bastante común

El papel del aislamiento reproductivo geográfico

La distribución geográfica de una especie viene definida por rasgos genéticos, evolutivos, históricos y/o ecológicos (Whittaker 1972; Comes & Kadereit 1998; Elith & Leathwick 2009; Sexton et al. 2009). A su vez, la presencia de poblaciones dentro de una especie con patrones de distribución diferenciados puede limitar el flujo génico favoreciendo el aislamiento genético y la diferenciación (Dobzhansky 1940; Irwin 2002; Anacker & Strauss 2014). Sin embargo, los procesos de migración intervienen directamente sobre el flujo génico dado que el aislamiento reproductivo geográfico no solo viene definido por la disposición física de las poblaciones, también esta determinado por los procesos de intercambio de diásporas entre núcleos poblacionales distanciados (Winker 2000; Nosil 2008; Delmore et al. 2015). En base al modelo de distribución de nicho, hemos estimado que en el 63% del área de distribución de *Lysimachia arvensis*, los dos linajes no coocurren en las poblaciones. Por tanto, la única posibilidad de flujo génico entre linajes en esas áreas tendría lugar

como consecuencia de intercambio de polen o semillas entre poblaciones. *L. arvensis* no presenta mecanismos de dispersión de polen o semillas a larga distancia, ya que es polinizada por abejas solitarias que construyen sus nidos en el suelo muy cerca de las plantas y las semillas se dispersan por barocoria. Por tanto, el flujo génico entre poblaciones debe ser posiblemente reducido. Es bien conocida la importancia del aislamiento geográfico en especiación (Mayr 1959) y un rango limitado de solapamiento geográfico entre especies relacionadas puede indicar que la este aislamiento tuvo un papel relevante iniciando el proceso de especiación (Barraclough & Vogler 2000). Los patrones de distribución que hemos encontrado, claramente asocian a cada linaje con unas preferencias ecológicas determinadas. El linaje azul se distribuye en ambientes mucho más secos y más sometidos a estrés hídrico estival que el linaje rojo, hecho que se había descrito previamente (Arista et al. 2013). Por tanto, podemos afirmar que la barrera geográfica entre linajes de *L. arvensis* es una barrera ecogeográfica (según Ramsey et al. 2003) y supuso un grado de aislamiento entre linajes bastante alto, sobre todo para el rojo que tiene un área de distribución mayor y por tanto se ve menos influenciado por la presencia del linaje azul. Además, según nuestro modelo de distribución, el linaje rojo muestra un área de distribución relativamente grande con zonas donde no hay poblaciones cercanas del linaje azul, por lo que en ellas el aislamiento debe ser absoluto. En la gran mayoría de los estudios de aislamiento reproductivo entre pares de taxones cercanos, la barrera geográfica no es considerada y solo se estudian aquellas que ocurren en simpatría (ver revisión en Baak et al. 2015). Sin embargo, nuestros resultados son un buen ejemplo de la importancia de incluir el estudio de la barrera geográfica como uno de los impedimentos al flujo génico lo que ha sido puesto de manifiesto en algunos trabajos recientes (Brys et al. 2014, Sobel & Streisfeld 2015; Runquist et al. 2014).

Barreras de aislamiento precigótico en poblaciones simpátricas.

La acción conjunta de tres barreras precigóticas (asincronía de floración, patrones de polinización y precedencia del polen) disminuyó la frecuencia esperada de producción de semillas híbridas en el linaje azul de *L. arvensis* en un 76,65% y en el linaje rojo en un 61,27%. En este último, encontramos que cada una estas barreras contribuyeron de manera similar a la formación de semillas híbridas, siendo más relevantes la asincronía de floración estacional y la precedencia polínica. Sin embargo, en el linaje azul el impedimento para la formación de semillas híbridas se debió casi en su totalidad a una única barrera reproductiva precigótica, la selección mediada por polinizadores. La asincronía en la floración es consecuencia de la eficiencia superior que muestra el linaje azul en la germinación, en ambientes mediterráneos, respecto del linaje rojo (capítulo 4). Esa diferencia se mantiene hasta el periodo de floración, en

la mayoría de las poblaciones estudiadas, donde las plantas del linaje rojo alcanzan el máximo de floración cuando las plantas de flores azules están próximas a la senectud (Fig. 2). En consecuencia, el apareamiento aleatorio entre plantas está fuertemente sesgado hacia el color mayoritario en cada caso, disminuyendo los cruces entre linajes significativamente. Por otro lado, las preferencias de los polinizadores por las flores azules de *L*. *arvensis* (Fig. 4), confirma los resultados de trabajos previos en ambientes mediterráneos (Arista et al. 2013), y como el tamaño floral y la recompensa de polen no son significativamente distintos entre linajes de la especie (Datos no mostrados), el color de la flor debe ser determinante en el atractivo para los polinizadores. Además, la eficiencia polínica fue significativamente distinta entre linajes. El polen del linaje rojo tuvo menor éxito del esperado en general, produciéndose proporciones de descendencia hibrida mayor de la esperada en el linaje rojo y menor de la esperada en el linaje azul, aunque en general la descendencia híbrida fue significativamente menor que la originada por cruces dentro de cada linaje (Fig. 5)

Como resultado de todas estas barreras el linaje azul fue más propenso a la deposición de polen heteromorfico en el linaje rojo, provocando mayor aislamiento del primer linaje. Patrones similares se observaron en *Centaurium littorale*/*C*. *erythraea* o *Mimulus guttatus*/*M*. *nasutus* donde la primera especie sufría una reducción en las deposiciones de polen heterospecífica después de la acción secuencial de estos componentes precigóticos (Martin & Willis 2007; Brys et al. 2014). En ambos casos, tanto *C*. *erythraea* como *M*. *nasutus* mostraron estrategias predominantemente autogamas, al igual que ocurre en el linaje rojo de *L*. *arvensis* (Capitulo 5). Las diferencias en el éxito de la deposición de polen heteroespecífico en las especies con mayor tasa de autofecundación pueden exacerbarse cuando la especie se encuentra en minoría en comparación con su pariente más efectivo, como se observa en nuestra especie de estudio. No obstante, los valores observados de resistencia a las barreras precigóticas sugieren que todavía existe una oportunidad para la producción potencial de semillas híbridas, por ello se han observado esporádicamente individuos del fenotipo salmón en poblaciones naturales (Capítulo 2) y hace necesario el estudio de las barreras postcigóticas para entender la escasez de flores de color salmón en la naturaleza.

Barreras de aislamiento postcigótico en poblaciones simpátricas.

A pesar de la gran cantidad de progenie híbrida F_1 que se obtuvo después de cruzar manualmente las flores de ambos linajes, las probabilidades de formación de semillas híbridas F_1, después de la acción secuencial de las cuatro barreras precigóticas estudiadas, fue pequeña (16,47% y 17,41% en los linajes rojo y azul,

respectivamente). Sin embargo, el aislamiento no es total, y dado que la descendencia de primera generación, del cruce entre plantas azules y rojas, siempre presenta un fenotipo de flores de color salmón (Capitulo 1), cabría esperar que dicho fenotipo se encontrara en poblaciones naturales en proporciones superiores a las observadas. Máxime considerando el éxito de algunos de los rasgos del fitness (seed-set parental y germinación) en plantas híbridas que muestra nuestros resultados, especialmente en un sentido del cruce (RxA) (Fig. 6). La escasez de observaciones del fenotipo salmón podría explicarse por varias razones. La primera es el efecto de ciertos rasgos del fitness que actúen como barreras al flujo génico, esto se observa en *L. arvensis* en la producción de polen, óvulos y, especialmente, semillas de la progenie híbrida respecto de sus congéneres isomórficas (Fig 6). Este detrimento del fitness se ha observado en otras especies de angiospermas como *Iris*, *Orchis*, *Mimulus*, o *Gossypium* (Burke et al. 1998; Fishman & Willis 2001; Jiang et al. 2000; Scopece et al. 2008) y puede haber surgido de irregularidades meióticas (Stebbins 1958; Rieseberg 2001) o por un mecanismo de incompatibilidad Dobzhansky-Muller (DMI) en el aislamiento reproductivo parcial de acción tardía en sucesivas generaciones (Stacy et al. 2017). El seed-set se redujo significativamente para la progenie híbrida (aprox. 40% más bajo que los dos linajes parentales), y en la F_2 se observaron plantas completamente estériles en *L. arvensis*. Este patrón de fertilidad híbrida reducida ya fue detectado en *Mimulus* por Fishman y Willis (2001) en su estudio comparativo de reordenamientos cromosómicos contra DMI como la causa de las barreras postcigóticas; el decremento en la fertilidad femenina de la progenie híbrida de segunda generación en comparación con los F_1 es consistente con la pérdida de fertilidad debido a los DMI diploides recesivos (Fishman & Willis 2001). A pesar que los patrones de germinación y supervivencia de las plántulas no apoyaron la hipótesis de DMI en los híbridos interlinaje de *L. arvensis*, es este mecanismo (DMI) la principal fuente genética de inestabilidad y esterilidad en híbridos (Coyne & Orr 2004; Stacy et al. 2017). Se considera que estos mecanismos de incompatibilidad aparecen como consecuencia de interacciones perjudiciales entre alelos de diferentes loci que han evolucionado de forma independiente en las poblaciones parentales (Coyne & Orr 2004; Gavrilets 2004).

Por otro lado, la ausencia de plantas híbridas puede estar relacionado con la capacidad del linaje o de la especie en una autopolinización competitiva, muy probablemente a través de una obstrucción del estigma por polen propio. Ello sería posible por el posicionamiento cercano de las anteras alrededor de la superficie estigmática evitando que los polinizadores entren en contacto directo con el estigma y maximiza el número de granos de polen propio depositados en el estigma (Webb & Lloyd 1986). Este fenómeno se observó en otras especies como *Ipomea hederacea*

(Smith & Rausher, 2007) donde la proximidad estigma-antera era una barrera de aislamiento precigótica importante cuando estas flores estaban expuestas al flujo de polen heteroespecífico de *Ipomea purpurea*. En *L. arvensis* encontramos alta variabilidad poblacional para los rasgos de hercogamia, aunque la tendencia es positiva en el linaje azul y negativa en el rojo. Solo un bajo grado de hercogamia lateral implica autofecundación predominante, y esto fue observado principalmente en el linaje azul cuando vive en simpatría con el linaje rojo (Capítulo 6). Este rasgo floral podría explicar las limitaciones al flujo polínico en poblaciones naturales y la carestía de observaciones del morfo salmón. Por otro lado, las especies con autofecundación en general producen polen con un menor crecimiento del tubo polínico y habilidades competitivas mermadas, pudiendo causar desventajas tras la deposición de cargas de polen mixto (Montgomery et al. 2010; Runquist 2012). En el caso de *L. arvensis*, desconocemos si el crecimiento del tubo polínico es significativamente más lento en el linaje rojo, pero el polen del linaje azul es más eficiente y sería concordante con la hipótesis anterior, donde especie con baja hercogamia lateral están adaptadas a la autopolinización. Además, los datos de aislamiento reproductivo, junto a los efectos de depresión por endogamia del linaje rojo (capitulo 4) si apoyan la estrategia de autofecundación en el linaje rojo.

Aislamiento total y Asimetría entre linajes de L. arvensis

A pesar de la acción de las 10 barreras estudiadas antes y después de la fecundación, el aislamiento reproductivo total todavía permaneció incompleto (RI_{total} = 0,7855). El hecho de que se han observado híbridos salmón en el campo está en línea con estos resultados. Varios estudios han descrito, basándose únicamente en las características morfológicas, la aparición de híbridos entre linajes de *L. arvenis* (Marsden-jones & Weiss 1938; Capitulo 2).

En especies estrechamente relacionadas, el aislamiento reproductivo rara vez se logra mediante la acción de una sola barrera reproductiva (Lowry et al. 2008) y la resistencia de las barreras a menudo parece ser asimétrica entre cruces recíprocos (Tiffin et al. 2001; Rieseberg & Willis 2007). En *L. arvensis* se observa una clara asimetría entre los linajes azul y rojo, especialmente cuando actúan como padres. El linaje azul, como padre, solo muestra un aislamiento ligeramente superior al 50% (RI= 0,5603) mientras que el rojo presenta un aislamiento casi completo (RI = 0,9114). En ambos casos el aislamiento es del 75-80% cuando actúan como madres. La asimetría entre cruces recíprocos de distintas especies ha sido ampliamente documentada (Harrison 1986; Coyne & Orr 1989; Gallant & Fairbairn 1997; Tiffin et al. 2001; Willett & Burton 2001; Presgraves 2002; Bolnick & Near 2005; Buggs 2007; Scascitelli et al. 2010; Palma-

Silva et al., 2011; Ruhsam et al. 2011) y es esperable dada la variación en los rasgos florales y las proporciones de ocurrencia espacial y/o temporal.

CONCLUSIONES

En las especies simpátricas que comparten una morfología floral generalizada y una comunidad de polinizadores, la intensa competencia por la polinización y los costes relacionados con la hibridación pueden seleccionar rasgos florales que contribuyen al aislamiento precigótico (Servedio & Noor 2003). Un rasgo claramente seleccionado, según nuestros resultados, es el color floral. En los linajes de *Lysimachia arvensis* estudiados, nuestras observaciones mostraron claramente que la transferencia de polen heteroespecífica y la fertilización híbrida eran costosas, tanto en términos de producción de semillas como en otros rasgos del fitness de la progenie. Bajo tales condiciones, las características morfológicas que evitan mecánicamente la transferencia de polen intraespecífica y favorecen la polinización cruzada (por ejemplo, hercogamia lateral) pueden favorecerse para minimizar el aislamiento Las investigaciones previas de algunas poblaciones simpátricas y alopátricas de ambos linajes en la región mediterránea mostraron diferencias significativas en la separación entre el estigma y la antera en *L. arvensis*, dependiendo de si los fenotipos florales coexistieron o no (Capítulo 6). Aunque el aislamiento no fue total, si que se ha observado un fuerte efecto de las barreras al flujo génico, especialmente en el linaje rojo. Y quizás *L. arvensis* pueda ser definido como un modelo de especiación incipiente, donde el efecto de las barreras aquí analizadas está contribuyendo a una diferenciación genética. En trabajos futuro debe explorarse el efecto de la diversidad genética en la distribución de la especie y sus posibles consecuencias filogenéticas. Asimismo, experimentos de selección pueden aplicarse con el fin de proporcionar evidencia de que el aislamiento precigótico mejora como respuesta a la selección frente a la hibridación. Dichos experimentos también podrían demostrar si cambios del sistema de reproducción se asocian con rasgos florales y mejoran las asimetrías en el flujo polínico, resultando un mayor aislamiento reproductivo total.

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REFERENCIAS

Anacker BL. & Strauss SY. (2014). The geography and ecology of plant speciation: range overlap and niche divergence in sister species. *Proceedings of the Royal Society of London B*, **281**: 20132980.

Arista M, Talavera M, Berjano R. & Ortiz PL. (2013). Abiotic factors may explain the geographical distribution of flower colour morphs and the maintenance of colour polymorphism in the scarlet pimpernel. *Journal of Ecology*, **101**: 1613–1622.

Baack E, Melo MC, Rieseberg LH. & Ortiz-Barrientos D. (2015). The origins of reproductive isolation in plants. *New Phytologist*, **207**: 968-984.

Barraclough TG. & Vogler AP. (2000). Detecting the geographical pattern of speciation from species-level phylogenies. *The American Naturalist*, **155**: 419-434.

Bolnick DI. & Near TJ. (2005). Tempo of hybrid inviability in centrarchid fishes (Teleostei: Centrarchidae). *Evolution*, **59**: 1754-1767.

Brys R, Broeck AV, Mergeay J. & Jacquemyn H. (2014). The contribution of mating system variation to reproductive isolation in two closely related *Centaurium* species (Gentianaceae) with a generalized flower morphology. *Evolution*, **68**: 1281-1293.

Buggs RJA. (2007). Empirical study of hybrid zone movement. *Heredity*, **99**: 301.

Burke JM, Voss TJ. & Arnold ML. (1998). Genetic interactions and natural selection in Louisiana *Iris* hybrids. *Evolution*, **52**: 1304–1310.

Comes HP. & Kadereit JW. (1998). The effect of Quaternary climatic changes on plant distribution and evolution. *Trends in plant science*, **3**: 432-438.

Coyne JA. & Orr HA. (1989). Patterns of speciation in *Drosophila*. *Evolution*, 43: **362**-381.

Coyne JA. & Orr HA. (2004). Speciation. Sunderland, MA: Sinauer Associates.

Cozzolino S. & Scopece G. (2008). Specificity in pollination and consequences for postmating reproductive isolation in deceptive Mediterranean orchids. *Philosophical Transactions of the Royal Society B*, **363**: 3037-3046.

Dell'Olivo A, Hoballah ME, Gübitz T. & Kuhlemeier C. (2011). Isolation barriers between *Petunia axillaris* and *Petunia integrifolia* (Solanaceae). *Evolution: International Journal of Organic Evolution*, **65**: 1979-1991.

Delmore KE, Kenyon HL, Germain RR. & Irwin DE. (2015). Phenotypic divergence during speciation is inversely associated with differences in seasonal migration. *Proceedings of the Royal Society B*, **282**: 20151921.

Dobzhansky T. (1937). Genetic nature of species differences. *The American Naturalist*, **71**: 404-420.

Dobzhansky T. (1940). Speciation as a stage in evolutionary divergence. *The American Naturalist*, **74**: 312-321.

Dobzhansky T. (1951). Genetics and the origin of species. Columbia Univ. Press

Elith J. & Leathwick JR. (2009). Species distribution models: ecological explanation and prediction across space and time. *Annual review of ecology, evolution, and systematics*, **40**: 677-697.

Fishman L. & Willis JH. (2001). Evidence for Dobzhansky-Muller incompatibilities contributing to the sterility of hybrids between *Mimulus guttatus* and *M. nasutus*. *Evolution*, **55**: 1932–1942.

Gallant SL. & Fairbairn DJ. (1997). Patterns of postmating reproductive isolation in a newly discovered species pair, *Aquarius remigis* and *A. remigoides* (Hemiptera; Gerridae). *Heredity*, **78**: 571.

Gavrilets S. (2004). Fitness landscapes and the origin of species (MPB-41). 41. Princeton University Press.

Gibbs P.E. & Talavera S. (2001). Breeding system studies with three species of *Anagallis* (Primulaceae): self-incompatibility and reduced female fertility in *A. monelli* L. *Annals of Botany*, **88**: 139-144.

Grant V. (1981). Plant speciation. Columbia University Press.

Harrison RG. (1986). Pattern and process in a narrow hybrid zone. *Heredity*, **56**: 337.

Hijmans RJ, Guarino L, Cruz M. & Rojas E. (2001). Computer tools for spatial analysis of plant genetic resources data: 1. DIVA-GIS. *Plant Genetic Resources Newsletter*, 15-19.

Hopkins R. (2013). Reinforcement in plants. *New Phytologist*, **197**: 1095-1103.

Hopkins R. & Rausher MD. (2014). The cost of reinforcement: selection on flower color in allopatric populations of *Phlox drummondii*. *The American Naturalist*, **183**: 693-710.

Howard DJ. (1999). Conspecific sperm and pollen precedence and speciation. *Annual Review of Ecology and Systematics,* **30**:109–32

Irwin DE. (2002). Phylogeographic breaks without geographic barriers to gene flow. *Evolution*, **56**: 2383-2394.

Jiang CX, Chee PW, Draye X, Morrell PL, Smith CW. & Paterson AH. (2000). Multilocus interactions restrict gene introgression in interspecific populations of polyploid *Gossypium* (cotton). *Evolution*, 54: 798–814.

Jiménez-López FJ, Arista M, Talavera M, Pannell JR. & Ortiz PL. (2019). Heritabilities of lateral and vertical herkogamy in *Lysimachia arvensis*. *Plant Species Biology*, **34**: 31-37.

Kay KM. (2006). Reproductive isolation between two closely related hummingbird pollinated neotropical gingers. *Evolution*, **60**: 538-552.

Lowry DB, Modliszewski JL, Wright KM, Wu CA. & Willis JH. (2008). The strength and genetic basis of reproductive isolating barriers in flowering plants. *Philosophical Transactions of the Royal Society B*, **363**: 3009-3021.

Mallet J. (2008). Hybridization, ecological races and the nature of species: empirical evidence for the ease of speciation. *Philosophical Transactions of the Royal Society of London B*, **363**: 2971-2986.

Marsden-Jones EM. & Weiss FE. (1938). The essential differences between *Anagallis arvensis* L. and *Anagallis foemina* Mill. – *Proceedings of the Royal Society of London B*, **150**: 146-155.

Martin NH. & Willis JH. (2007). Ecological divergence associated with mating system causes nearly complete reproductive isolation between sympatric *Mimulus* species. *Evolution,* **61**: 68–82.

Mayr E. (1942) Systematics and the origin of species, from the viewpoint of a zoologist. Harvard University Press.

Mayr E. (1959). Isolation as an evolutionary factor. *Proceedings of the American Philosophical Society*, **103**: 221-230.

Montgomery BR, Soper DM. & Delph LF. (2010). Asymmetrical conspecific seed-siring advantage between *Silene latifolia* and *S. dioica*. *Annals of Botany*, **105**: 595-605.

Muller H. (1942). Isolating mechanisms, evolution, and temperature. *In Biol. Symp*. **6**: 71-125.

Noor MA. (1997). How often does sympatry affect sexual isolation in *Drosophila*?. *The American Naturalist*, **149**: 1156-1163.

Nosil P. (2008). Speciation with gene flow could be common. *Molecular ecology*, **17**: 2103-2106.

Nosil P. (2012). Ecological speciation. Oxford University Press.

Nosil P. & Crespi BJ. (2006). Ecological divergence promotes the evolution of cryptic reproductive isolation. *Proceedings of the Royal Society of London B*, **273**: 991-997.

Nosil P, Vines TH. & Funk DJ. (2005). Reproductive isolation caused by natural selection against immigrants from divergent habitats. *Evolution*, **59**: 705-719.

Ortiz PL, Berjano R, Talavera M, Rodríguez-Zayas L. & Arista M. (2015). Flower colour polymorphism in *Lysimachia arvensis*: how is the red morph maintained in Mediterranean environments? *Perspective Plant Ecology, Evolution & Systematics*, **17**: 142– 150.

Palma-Silva C, Wendt T, Pinheiro F, Barbará T, Fay MF, Cozzolino S. & Lexer C. (2011). Sympatric bromeliad species (*Pitcairnia* spp.) facilitate tests of mechanisms involved in species cohesion and reproductive isolation in Neotropical inselbergs. *Molecular Ecology*, **20**: 3185-3201.

Presgraves DC. (2002). Patterns of postzygotic isolation in Lepidoptera. *Evolution*, **56**: 1168-1183.

Raine NE. & Chittka L. (2007). The adaptive significance of sensory bias in a foraging context: floral colour preferences in the bumblebee *Bombus terrestris*. *PLoS One*, 2: e556.

Ramsey J, Bradshaw HD. & Schemske DW. (2003). Components of reproductive isolation between the monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). *Evolution*, **57**: 1520-1534.

Rieseberg LH. (2001). Chromosomal rearrangements and speciation. *Trends in ecology & evolution*, **16**: 351-358.

Rieseberg LH. & Willis JH. (2007). Plant speciation. *Science*, **317**: 910-914

Ruhsam M, Hollingsworth PM. & Ennos RA. (2011). Early evolution in a hybrid swarm between outcrossing and selfing lineages in *Geum*. *Heredity*, **107**: 246.

Rundle HD, Nagel L, Boughman JW. & Schluter D. (2000). Natural selection and parallel speciation in sympatric sticklebacks. *Science*, **287**: 306-308.

Runquist RDB. (2012). Pollinator-mediated competition between two congeners, *Limnanthes douglasii* subsp. *rosea* and *L. alba* (Limnanthaceae). *American journal of botany*, **99**: 1125-1132.

Runquist RD, Chu E., Iverson J.L., Kopp J.C. & Moeller D.A. (2014). Rapid evolution of reproductive isolation between incipient outcrossing and selfing *Clarkia* species. *Evolution*, **68**: 2885-2900.

Scascitelli M, Whitney KD, Randell RA, King M, Buerkle CA. & Rieseberg LH. (2010). Genome scan of hybridizing sunflowers from Texas (*Helianthus annuus* and *H. debilis*) reveals asymmetric patterns of introgression and small islands of genomic differentiation. *Molecular Ecology*, **19**: 521-541.

Schemske DW. (2000). Understanding the origin of species 1. *Evolution*, **54**: 1069-1073.

Schluter D. (2000). Ecological character displacement in adaptive radiation. *The american naturalist*, **156**: S4-S16.

Scopece G, Widmer A. & Cozzolino S. (2008). Evolution of postzygotic reproductive isolation in a guild of deceptive orchids. *The American Naturalist*, **171**: 315–326.

Servedio MR. & Noor MA. (2003). The role of reinforcement in speciation: theory and data. *Annual Review of Ecology, Evolution, and Systematics*, **34**: 339-364.

Sexton JP, McIntyre PJ, Angert AL. & Rice KJ. (2009). Evolution and ecology of species range limits. *Annual Review Ecology Evolution Systematics*, **40**: 415-436.

Smith RA. & Rausher MD. (2007). Close clustering of anthers and stigma in *Ipomoea hederacea* enhances prezygotic isolation from *Ipomoea purpurea*. *New Phytologist*, **173**: 641-647.

Sobel JM. & Streisfeld MA. (2015). Strong premating reproductive isolation drives incipient speciation in *Mimulus aurantiacus*. *Evolution*, **69**: 447-461.

Sobel JM. & Chen GF. (2014). Unification of methods for estimating the strength of reproductive isolation. *Evolution*, **68**: 1511-1522.

Stacy EA, Paritosh B, Johnson MA. & Price DK. (2017). Incipient ecological speciation between successional varieties of a dominant tree involves intrinsic postzygotic isolating barriers. *Ecology and evolution*, **7**: 2501-2512.

Stebbins GL. (1958). The inviability, weakness, and sterility of interspecific hybrids. *In Advances in genetics,* **9**: 147-215. Academic Press.

Phillips SJ, Dudík M. & Schapire RE. [Internet] Maxent software for modeling species niches and distributions (Version 3.4.1). Available from url: http://biodiversityinformatics.amnh.org/open_source/maxent/. Accessed on 2018-11-26.

Tiffin P, Olson S. & Moyle LC. (2001). Asymmetrical crossing barriers in angiosperms. *Proceedings of the Royal Society of London B*, **268**: 861-867.

Webb CJ. & Lloyd DG. (1986). The avoidance of interference between the presentation of pollen and stigmas in Angiosperms. II. Herkogamy. *New Zealand Journal of Botany*, **24**: 163–178.

Whittaker RH. (1972). Evolution and measurement of species diversity. Taxon, 213-251.

Willett CS. & Burton RS. (2001). Viability of cytochrome c genotypes depends on cytoplasmic backgrounds in *Tigriopus californicus*. *Evolution*, **55**: 1592-1599.

Winker K. (2000). Evolution: migration and speciation. *Nature*, **404**: 36.

8. Phylogeny and taxonomic implications for the Mediterranean *Anagallis*, currently in *Lysimachia*.

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ABSTRACT

Anagallis arvensis and *A. monelli* are very variable species and a wide number of infraspecific taxa have been described within each one. Flower colour polymorphism joins this taxonomic complexity because both species have plants with blue or red flowers. Implications of colour polymorphism of both species have not received enough attention in phylogenetic studies. We defined the phylogenetic identity of the blue and red morphs of each species and we proposed taxonomic implications. To phylogenetic construction, plastid markers (*rps16*-*trnK*, rpl32-*trnL*, *trnH*-*psbA*) and ITS were used. Our results show that colour morphs of *Lysimachia arvensis* (former *Anagallis arvensis*) and *L. monelli* (former *Anagallis monelli*) appear together with plastid markers but they are split with nuclear marker. Therefore our results with nuclear marker reveal a significant association among each colour morph of *L. arvensis* and *L. monelli*. In addition, blue morph of *L. arvensis* is brother of *L. talaverae*, red morph of *L. arvensis* is brother of red morph of *L. monelli* and *L. foemina* is brother of blue morph of *L. monelli*. The current isolation of each morph of each species is evident, although the common or independent origin of each requires more studies. Thus, we propose a new name for plants with blue flowers of *L. arvensis* (*L. loeflingii*) and a new combination for plants with red flowers of *L. monelli* (*L. collina*).

KEYWORDS

Nuclear and plastid DNA phylogenies, flower colour polymorphism, *Anagallis*, speciation, taxonomy

INTRODUCTION

The tribe Lysimachieae (Benth. & Hook. f.) Pax (Primulacea) is formed by seven genera: *Glaux* L., *Lysimachia* L., *Trientalis* L., *Asterolinum* Hoffmanns. Link, *Pelletiera* A. St. Hil., *Anagallis* L. and *Centunculus* L., all with capsules with valvicidal dehiscence, except *Anagallis* and *Centunculus* that the capsule is opened by a transverse or circumcised suture. The systematic circumscription of the taxa belonging to the *Lysimachia* complex has been thoroughly studied since Linnaeus descriptions (Harborne, 1968; Källersjö et al., 2000; Martins et al., 2003; Hao et al., 2004; Manns & Anderberg, 2007b; Zhang et al., 2012). Traditionally, *Anagallis* L. (including *Centunculus*) has always been considered a genus closely related to *Lysimachia* L. In the revision of the genus *Anagallis* L. in the tropics and southern Africa, Taylor (1955) classified the species into three subgenera: *Anagallis* L. subg. *Anagallis* (5 species from Mediterranean Basin: *A. arvensis* L. (*A. arvensis* subsp. *arvensis* and *Anagallis arvensis* subsp. *foemina* (Mill.) Schinz & Thellung), *A. monelli* L., *A. parviflora* Hoffmanns. & Link, *Anagallis collina* Schousb., and *A. platyphylla* Baudo); *Anagallis* subg. *Jirasekia* (Smidt) P. Tayl. (11 species from South Africa, south America and West of Mediterranean basin); and *Anagallis* subg. *Centunculum* (L.) P. Tayl. (8 species from South Africa). Analysis of ITS and plastid data showed that the studied species of *Anagallis* L. subg. *Anagallis* (*Anagallis arvensis*, *A. monelli and A. foemina*) were more closely related to species of genera *Pelletiera*, *Asterolinon*, and *Lysimachia nemorum* and *L. serpyllifolia* than to species of *Anagallis* subg. *Jirasekia* and *Anagallis* subg. *Centunculum* (Manns & Anderberg, 2005; Manns & Anderberg., 2007b). It entitled the inclusion of these taxa within *Lysimachia* (Manns & Anderberg, 2007b, 2009). In accordance with the results of phylogenetic analysis, new combinations were proposed to these species of *Anagallis* L. subg. *Anagallis*: *Lysimachia arvensis* (L.) U. Manns & Anderb., *L. monelli* (L.) U. Manns & Anderb. and *L. foemina* (Mill.) U. Manns & Anderb. (Manns & Anderberg, 2009).

The high diversity existent within *Anagallis* L. subg. *Anagallis*, in which morphological traits like leaf shape, flower colour, size and anatomy of glands in petals, difficult the delimitation of taxa. *Anagallis arvensis* and *A. monelli* show a high morphological and ecological diversity, and as consequence a wide number of infraspecific taxa has been described within each species. For example, within *A. arvensis* s.l, plants with small size and small blue flowers, particular ecology (Bolòs & Vigo, 1996; Gibbs & Talavera, 2001) and low level of ploidy (2n=20; Šveřepová, 1972; Kress, 1969; Talavera & al., 1997) were described as *Anagallis parviflora* Hoffmanns. & Link (Hoffmannsegg & Link, 1813-1820) or as infraspecific taxa of *Anagallis arvensis*. Recently, in basis on those traits, a recent study recognized this taxon as an independent species, *L. talaverae* L.

Saéz & Aymerich (Aymerich & Sáez, 2015). Likewise, plants morphologically very similar to *A. arvensis* but with blue flowers and four cells in the marginal glands of the petals (instead of three) were recognized as *A. arvensis* subsp. *foemina* (Mill.) Schinz & Thell., but a molecular study has suggested that this taxon is a different species [*Lysimachia foemina* (Mill.) U. Manns & Anderb.= *Anagallis foemina* Mill.] closer to *L. monelli* than to *L. arvensis* (Manns & Anderberg, 2007a). Moreover, annual plants with large blue flowers were described as *Anagallis platyphylla* Baudo. A similar situation happened in *Anagallis monelli*, for example, plants with narrow and linear leaves were described as *A. linifolia* L. (or *A. monelli* var. *linifolia* (L.) Lange). Plants with small and wide leaves and short internodes as *A. linifolia* var. *maritima* Mariz (or *A. monelli* subsp. *maritima* (Mariz) M. Lainz) or *A. linifolia* var. *microphylla* Ball (or *A. monelli* var. *microphylla* (Ball.) Vasc.).

Lysimachia arvensis and *L. monelli* show a distinctive reproductive trait with two states: blue and red morphs of flowers (Ferguson, 1972; Pujadas, 1997), which increases the taxonomic complexity due to the numerous taxa described in relation to this trait. The original description of *A. arvensis* was based on a plant with red flowers (Linnaeus, 1753). The blue morph was first described as *A. latifolia* L. (Linnaeus, 1753), but later recognized as *A. arvensis* subsp. *latifolia* (L.) Arcang. (Arcangeli, 1894) or *Anagallis arvensis* L. subsp. *arvensis* var. *caerulea* auct. plur. Blue and red plants of *L. arvensis* show a marked geographical distributional pattern, pure blue or mixed populations appear in dryer Mediterranean localities while pure red populations predominate in more temperate Oceanic areas (Arista et al., 2013). The morphs differ in other traits as flower phenology or type of herkogamy (Chapter 7 & 5 respectively). In the case of *A. monelli*, the description was based on a blue flowered specimen (Linnaeus, 1753), and red individuals were first described by Schousboe (1800) as *A. collina* Schousb. or infraspecific taxa. Populations of *A. monelli* are basically monomorphic, with blue-flowered plants restricted to the Iberian Peninsula and NW of Africa while the red-flowered plants grow mainly in NW of Morocco, Sardinia and northeast of Spain (Willkomm, 1870).

Previous phylogenetics studies have scarcely explored the potential implications of colour polymorphism in taxa delimitation, because it was considered as part of the intraspecific variation and usually, only one of the morphs was included in the molecular analyses (Martins et al., 2003; Manns & Anderberg, 2005; Anderberg et al., 2007; Manns & Anderberg, 2007b). However, Manns & Anderberg (2007a) included the two morph types of *L. arvensis* from the same population, and both samples were in the same clade to rpl16, rps16, trnL-F and ndhF consensus tree in spite of the same two samples appeared separated to ITS reconstruction. This suggests that the two

flower colour morphs of *L. arvensis* and *L. monelli* need to be included in phylogenetic studies to clarify the taxa relationships.

In this context, our study aims to (1) define the phylogenetic identity of the blue and red morphs of *Lysimachia arvensis* and *L*. *monelli*, and establish the relationship of each morph between them and with their phylogenetic brothers *L*. *foemina* and *L. talaverae* (2) propose new names or combinations in accordance with the results of phylogenetic analyses and propose the lectotype of these taxa.

MATERIAL & METHODS

Plant material

Lysimachia arvensis, with blue and red flowers, is a Mediterranean species that at present is distributed around the world; it is annual, self-compatible (Gibbs & Talavera, 2001) and with 2*n*=40 (revised in Pastor, 1992; Talavera et al., 1997). *L. monelli,* with blue and red flowers, is distributed exclusively in the W of Mediterranean Region; it is perennial, self-incompatible (Gibbs & Talavera, 2001; Talavera et al., 2001; Freyre & Griesbach, 2004) and with 2*n*=20 chromosomes (Kress, 1969; Valdés, 1970; Šveřepová, 1972; Talavera et al., 1997). *L. foemina* is also a Mediterranean species but, as same as *L. arvensis*, at present is distributed around the world; it is annual, self-compatible (Marsden-Jones, 1935; Marsden-Jones & Weiss, 1938) and with 2*n*=40 (revised in Pastor et al., 1992). *L. talaverae* is endemic to the western Mediterranean, it is annual, self-compatible (Gibbs & Talavera, 2001) and with 2*n*=20 (Šveřepová, 1972; Talavera et al., 1997).

Freshly leaf material from 30 populations of *Lysimachia arvensis,* 12 of *L*. *monelli* (Fig. 1), 3 of *L*. *foemina* and 4 of *L. talaverae* (Table 1) were dried in silica-gel (Chase & Hills, 1991) for posterior molecular analysis. The sampling represented 9 red/14 blue pure populations and 7 mixed (red and blue) populations of *L. arvensis*. Populations of *L. monelli* included 4 red pure and 8 blue pure populations. Also, *Lysimachia azorica* Hornem. ex Hook. (1 pop.), *Asterolinon linum-stellatum* (L) Hoffmanns. & Lik (2 pops.), *Lysimachia tyrrhenia* U. Manns & Anderb. (former *Anagallis crassifolia* Thore, GenBank Accession No: AY855136) and *Lysimachia tenella* (L.) U. Manns & Anderb. (former *Anagallis tenella* (L.) L., GenBank Accession No: AY855150) were included as outgroups to root the ITS phylogenetic tree (Table 1).

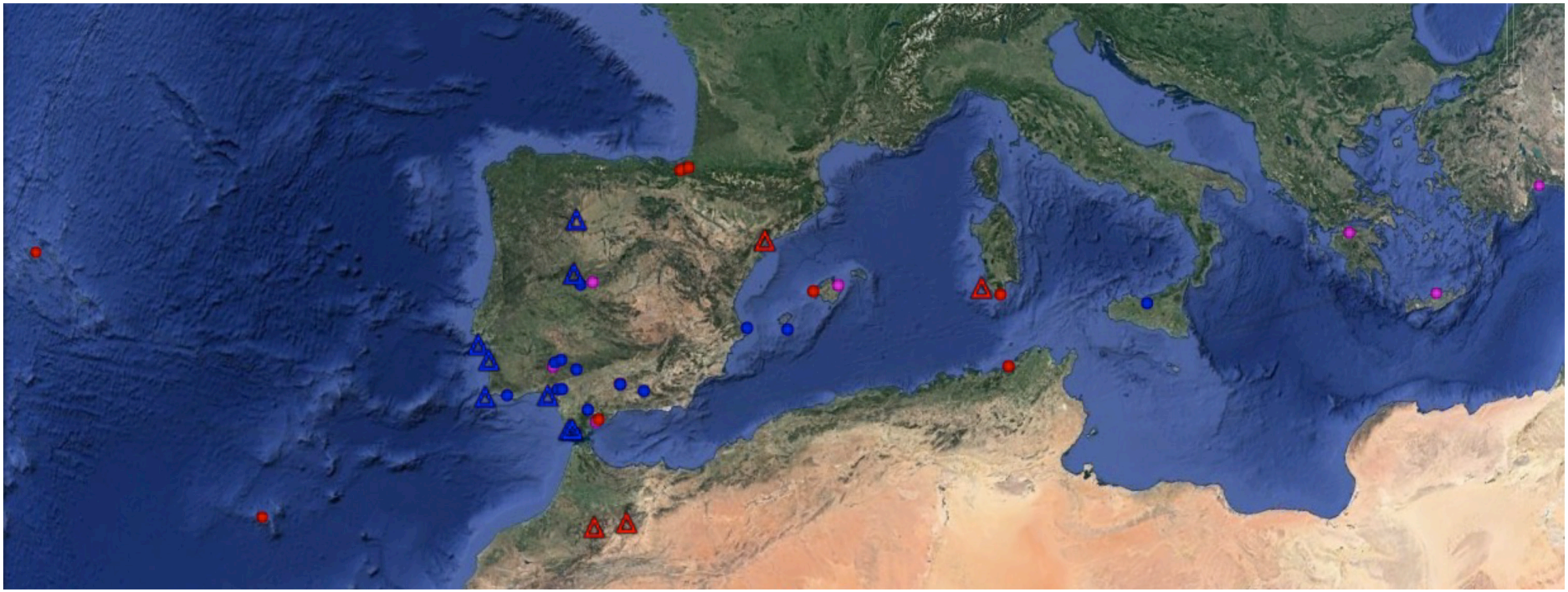

Figure 1. Geographical distribution of the studied populations of *Lysimachia arvensis* (circle) and *Lysimachia monelli* (triangle). The colours correspond to flower colour of the populations. Blue and red for pure blue and red populations, respectively, and pink for mixed populations were used.

Table 1. Population identity. For each population is shown country, region, coordinates, flower colour, herbarium code and accession numbers of Genbank database. ID: species and population code. Colour: (B) Blue, (R) Red , (Y) Yellow, (W) White.

ID	Colour		Locality	Coordinates	Voucher No.	Genbank Acc. No.			
						ITS	trnH-psbA	rps16-trnK	rpl32-trnL
Lysimachia arvensis **(L.) U. Manns & Anderb.**									
LA1	R	PRT	Azores. Sao Jorge	38°40'35.6"N-28°06'40.8"W	SEV275603	xxx	xxx	xxx	xxx
LA2	B	PRT	Albufeira. Praia de Falesia	37°5'12"N-8°10'9"W	SEV250700	xxx	xxx	xxx	xxx
LA3	R	PRT	Madeira. Boaventura	32°48'55"N-16°58'05"W	SEV285615	xxx			
LA4	B	ESP	Huelva. Hinojos	37°17'42.6"N-6°25'26.2"W	SEV279168	xxx	xxx	xxx	xxx
LA5	B	ESP	Huelva. Aracena	37°54'17.6"N-6°34'3.8"W	SEV278862	xxx	xxx	xxx	xxx
LA5	R	ESP	Huelva. Aracena	37°54'17.6"N-6°34'3.8"W	SEV278867		xxx	xxx	xxx
LA6	B	ESP	Huelva. Cañaveral de León	38°00'50.1"N-6°31'22.9"W	SEV279143	xxx			
LA7	B	ESP	Badajoz. Monesterio	38°5'26.9"N-6°17'9.6" W	SEV279171	xxx	xxx	xxx	xxx
LA8	B	ESP	Sevilla. Aznalcázar	37°18'09.9"N-6°15'45.4"W	SEV250703	xxx	xxx	xxx	xxx
LA9	B	ESP	Cádiz. Zahara de los Atunes	36°06'23.9"N-5°49'34.3"W	SEV279208	xxx			
LA10	R	ESP	Navarra. Leitza	43°05'1.04"N-1°54'59.16"W	SEV279272	xxx			
LA11	B	ESP	Sevilla. El Pedroso	37°50'16.3"N-5°45'41.9"W	SEV279228				
LA12	B	ESP	Cáceres. Losar de la Vera	40°06'38.0"N-5°34'56.2"W	SEV278765		xxx	xxx	xxx
LA13	B	ESP	Cádiz. Grazalema	36°45'25.1"N-5°23'42.4"W	SEV279114		xxx	xxx	xxx
LA14	B	ESP	Ávila. Poyales del Hoyo	40°10'32.6"N-5°09'30.7"W	SEV278770		xxx	xxx	xxx
LA14	R	ESP	Ávila. Poyales del Hoyo	40°10'32.6"N-5°09'30.7"W	SEV278773		xxx	xxx	xxx
LA15	B	ESP	Málaga. Estepona	36°25'44.81"N-5°8'4.76"W	-	xxx	xxx	xxx	xxx
LA15	R	ESP	Málaga. Estepona	36°25'44.81"N-5°8'4.76"W	SEV285214		xxx	xxx	xxx

ID	Colour		Locality	Coordinates	Voucher No.	Genbank Acc. No.			
						ITS	trnH-psbA	rps16-trnK	rpl32-trnL
LA16	R	ESP	Málaga. San Pedro de Álcántara	36º29'32.2"N-5º2'27.3"W	SEV286473	xxx			
LA17	R	ESP	Córdoba. Carcabuey. Fuente Dura	37°27'03.8"N-4°16'44.3"W	SEV279258	xxx	xxx	xxx	xxx
LA18	B	ESP	Córdoba. Carcabuey	37º26'23.3"N-4º16'37"W	SEV279276	xxx			
LA19	B	ESP	Granada. Sierra de Huétor	37º15'17"N-3º29'8"W	SEV279149				
LA20	B	ESP	Mallorca. Parc Natural de Levant	39º44'10.5"N-3º20'5.5"E	SEV279240	xxx	xxx	xxx	xxx
LA20	R	ESP	Mallorca. Parc Natural de Levant	39º44'10.5"N-3º20'5.5"E	SEV279236	xxx	xxx	xxx	xxx
LA21	B	ESP	Alicante. Denia	38º49'2.6" N-0º6'26.7" E	SEV278776	xxx			
LA22	R	ESP	Navarra. Oieregi	43°08'18.3"N-1°37'12.4"W	SEV279266	xxx	xxx	xxx	xxx
LA23	B	ESP	Formentera. Es Ca Mari		SEV252540	xxx			
LA24	R	ESP	Mallorca. Boal des Ses Severes	39º38'45.9"N-2º27'49.7"E	SEV256604-1	xxx			
LA25	R	ITA	Sardinia. Chia	38º53'38.8"N- 8º51'3.1"E	SEV252545	xxx	xxx	xxx	xxx
LA26	B	ITA	Sicily. Scillato-Caltavuturo	37º50'34.4"N-13º54'14.3"E	SEV279201	xxx			
LA27	R	TUN	Tabarka. Close to Algerian Frontier	36º57'46.4"N-8º44'51"E	SEV285272	xxx	xxx	xxx	xxx
LA28	B	GRC	Trapeza. Kalávrita	38°02'08.3"N-22°06'50.5"E	-	xxx	xxx	xxx	xxx
LA28	R	GRC	Trapeza. Kalávrita	38°02'08.3"N-22°06'50.5"E	-	xxx	xxx	xxx	xxx
LA29	B	GRC	Crete. Aglhia Pelaghia	35º24'36"N-24º59'51"E	SEV279241	xxx	xxx	xxx	xxx
LA29	R	GRC	Crete. Aglhia Pelaghia	35º24'36"N-24º59'51"E	SEV279244	xxx	xxx	xxx	xxx
LA30	B	TUR	Antalya. Belek	36º50'54.5"N-31º4'39.2"E	SEV252544-2	xxx	xxx	xxx	xxx
LA30	R	TUR	Antalya. Belek	36º50'54.5"N-31º4'39.2"E	SEV252544-1	xxx	xxx	xxx	xxx

ID	Colour		Locality	Coordinates	Voucher No.	Genbank Acc. No.			
						ITS	trnH-psbA	rps16-trnK	rpl32-trnL
Lysimachia monelli **(L.) U. Manns & Anderb.**									
LM1	B	PRT	Estremadura. Cabo Espichel	38º25'8"N-9º12'58"W	SEV284843	xxx	xxx	xxx	xxx
LM2	B	PRT	Algarve. Sagres	37º0'20"N-8º56'46"W	SEV284397	xxx			
LM3	B	PRT	Alentejo Litoral. Sines	38º0'55.2"N-8º49'11.1"W	SEV284791	xxx			
LM4	B	ESP	Huelva. Mazagón	37º7'26.3"N-6º45'46.2"W	SEV285034	xxx	xxx	xxx	xxx
LM5	B	ESP	Cádiz. Zahora	36º12'11.1"N-6º3'4.9"W	SEV286470	xxx			
LM6	B	ESP	Cádiz. Barbate	36º12'21.1"N-5º56'14.3"W	SEV286469	xxx			
LM7	B	ESP	Salamanca.Béjar	40º23'47"N-5º49'29"W	SEV285022	xxx			
LM8	B	ESP	Zamora. Granja de Moreruela	41º49'49"N-5º43'53"W	SEV284982	xxx	xxx	xxx	xxx
LM9	R	ESP	Tarragona. Mont Roig del Camp	41º5'18"N-0º56'5"E	SEV285107	xxx			
LM10	R	MAR	El Hajeb-Azrou	33°31'40.0"N-5°18'25"W	SEV218069	xxx	xxx	xxx	xxx
LM11	R	MAR	Fès. J. Bou- Iblane	33º39'37.1"N-4º14'5.1"W	SEV227757	xxx	xxx	xxx	xxx
LM12	R	ITA	Sardinia. Cala Fico	39º9'17.2"N-8º13'42.5"E	SEV252550	xxx	xxx	xxx	xxx
Lysimachia foemina **(Mill.) U. Manns & Anderb.**									
LF1	B	ESP	Almeria. Seron	37°20'55.8"N-2°30'35.3"W	SEV269286	xxx	xxx	xxx	xxx
LF2	B	ESP	Málaga. Almargen- Cañete la Real	36°58'6.56"N-5°2'33.18"W	SEV285199	xxx			
LF3	B	ESP	Mallorca. Boal des Ses Severes	39º38'45.9"N-2º27'49.7"E	SEV256604-2	xxx			
Lysimachia talaverae **L. Sáez & Aymerich**									
LT1	B	ESP	Huelva. Almonte	37°08'39.9"N-6°32'57.6"W	SEV286468	xxx	xxx	xxx	xxx
LT2	B	ESP	Huelva. Hinojos	37º17'37"N-6º25'15"W	SEV286467	xxx	xxx	xxx	xxx
LT3	B	PRT	Algarve. Vila do Bispo	37º7'18"N-8º53'39"W	SEV284451	xxx			

ID	Colour		Locality	Coordinates	Voucher No.	Genbank Acc. No.			
						ITS	trnH-psbA	rps16-trnK	rpl32-trnL
LT4	B	PRT	Ribatejo. Coruche	38º50'8"N-8º33'31"W	SEV284877	xxx			
Lysimachia azorica **Hornem. ex Hook.**									
LZ1	Y	PRT	Azores. Terceira. Algar do Carvao	38º43'54.4"N-27º19'16.7"W	SEV275597	xxx	xxx	xxx	xxx
Lysimachia linum-stellatum (**L.) Duby**									
LLS1	W	ESP	Sevilla. Dos Hermanas	37°21'06.5"N-5°56'21.9"W	SEV286828	xxx			
LLS1a	W	ESP	Sevilla. Dos Hermanas	37°21'06.5"N-5°56'21.9"W	SEV286828	xxx			
Lysimachia tyrrhenia **U. Manns & Anderb. (former *Anagallis crassifolia* Thore)**									
			XXX		GenBank Acc. No: AY855136				
Lysimachia tenella **L. (former *Anagallis tenella* L.)**									
			XXX		GenBank Acc. No: AY855150				

DNA Analysis

DNA isolation, amplification and sequencing

Total genomic DNA was isolated with Invisorb Vegetal DNA Kit HTS 96 (Invitek, Spain), with modifications following Jiménez-López et al. (2016). The quality of the extracted DNA was checked on 1% TAE-agarose gel, and the average DNA concentration was estimated photometrically using a NanoDrop DS-11 Spectrophotometer (DeNovix).

To phylogenetic construction, both ITS and plastid markers were used. Ribosomal ITS region 18S–5.8S–28S, were amplified using primers ITS5 (5' GGAAGGAGAAGTCGTAACAAGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') published by White et al. (1990). We tested twenty plastid markers (Table 2) in 8 samples of *L*. *arvensis* (4 blue and 4 red), but only three of them showed consistent polymorphism in the samples studied. The plastid markers *rps16–trnK*, *rpl32-trnL* amplified following Shaw et al. (2007) and *trnH–psbA* amplified following Hamilton (1999). The amplification was performed following Jiménez-López et al. (2016) using 4ng of DNA and the amplified fragments were checked on 2% TAE-agarose gels. An Applied Biosystems VeritiTM Thermal Cycler was used with the following programmes: for *rps16-trnK* and *rpl32-trnL*, first step (1 cycle) 80 °C/5 min; second step (35 cycles) 95 ºC/1 min, 50-65 ºC/1 min, 65ºC/3 min third step (1 cycle) 65 ºC/5 min and 4 ºC. For *trnH-psbA* the conditions were first step (1 cycle) 96 °C/5 min; second step (35 cycles) 95 ºC/45 sec, 53 ºC/1 min, 72ºC/30 sec third step (1 cycle) 72 ºC/5 min and 4 ºC. After purification with ExoSAP-IT (Roche, Spain), fragments were sequenced in an ABI 3730 machine, BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), at STAB Vida Lda. (Oeiras, Portugal). Sequences were edited with Geneious R10 (Biomatters Ltd, Auckland, New Zealand). Alignments were conducted in MEGA ver. 6 (Tamura et al., 2013) using the algorithm Clustal IW (Thompson et al., 2002).

Phylogenetic inference and dating

The evolution model which fit better to each of the regions was chosen with jModeltest 2.1.4 (Darriba et al., 2012), following the Akaike information criterion (AIC). The phylogenetic relationships were inferred with maximum parsimony using the software PAUP ver. 4.0b 10 (Swofford, 2003) and maximum likelihood methods with RAxML Ver. 8 (Stamatakis, 2014). Bootstrap proportion (BP) for each node was estimated with 500,000 bootstrap replicates following full heuristic searches. Bayesian analyses were conducted using the software Beast v1.8.4 (Drummond et al., 2012). For each analysis,

Table 2. Primer combinations tested. Tan = temperature of annealing. In bold primer combination selected.

NAME	SEQUENCE	SOURCE	TAN(ºC)	PCR CONDITION
trnK-3914F	GGG GTT GCT AAC TCA ACG G	Ohsako et al. 200	50	96º(1')/Tan(2')/72º(3')/72º(7')
trnK-2R	AAC TAG TCG GAT GGA GTA G			
trnC5'-R	TGC CTT ACC ACT CGG CCA T	Ohsako et al. 200	50	96º(1')/Tan(2')/72º(3')/72º(7')
rpoB5'-R	GTA GAT ATT CCC TCA TTT CC			
rpS16x2F2	AAA GTG GGT TTT TAT GAT CC	Shaw et al. 2007	50-65	80º(5')/95º(1')/50º(+0,3º-ciclo)-65º(1')/65º(4')/65º(5')
trnK(UUU)x1	TTA AAA GCC GAG TAC TCT ACC			
trnL(UAG)	CTG CTT CCT AAG AGC AGC GT	Shaw et al. 2007	50-65	80º(5')/95º(1')/50º(+0,3º-ciclo)-65º(1')/65º(4')/65º(5')
rpL32-F	CAG TTC CAA AAA AAC GTA CTT C			
trnH	ACG GGA ATT GAA CCC GCG CA	Demesure 1995	62	94º(4')/92º(45")/Tan(45")/72º(3')/72º(10')
trnK	CCG ACT AGT TCC GGG TTC GA			
trnK1	GGG TTG CCC GGG ACT CGA AC	Demesure 1995	53,5	94º(4')/92º(45")/Tan(45")/72º(3')/72º(10')
trnK2	CAA CGG TAG AGT ACT CGG CTT TTA			
trnD	ACC AAT TGA ACT ACA ATC CC	Demesure 1995	54,5	94º(4')/92º(45")/Tan(45")/72º(3')/72º(10')
trnT	CTA CCA CTG AGT TAA AAG GG			
psbC	GGT CGT GAC CAA GAA ACC AC	Demesure 1995	57	94º(4')/92º(45")/Tan(45")/72º(3')/72º(10')
trnS	GGT TCG AAT CCC TCT CTC TC			
trnSf	GAG AGA GAG GGA TTC GAA CC	Demesure 1995	62	94º(4')/92º(45")/Tan(45")/72º(3')/72º(10')
trnfM	CAT AAC CTT GAG GTC ACG GG			
psbAF	GTT ATG CAT GAA CGT AAT GCT C	Sang 2007	52	80º(5')/94º(30")/52º(30")/72º(1')72º(9')
trnHR	CGC GCA TGG TGG ATT CAC AAA TC			

NAME	SEQUENCE	SOURCE	TAN(ºC)	PCR CONDITION
rpL32-R	CCA ATA TCC CTT CCT TTT CCA A	Shaw et al. 2007	50-65	80º(5')/95º(1')/50º(+0,3º-ciclo)-65º(1')/65º(4')/65º(5')
ndhF	GAA AGG TAT TAT CCA CGM ATA TT			
trnC	CCA GTT CAA ATC TGG GTG TC	Demesure 1995	58	94º(4')/92º(45")/Tan(45")/72º(3')/72º(10')
trnD	GGG ATT GTA GTT CAA TTG GT			
psaA	ACT TCT GGT TCC GGC GAA CGA A	Demesure 1995	58	94º(4')/92º(45")/Tan(45")/72º(3')/72º(10')
trnS	AAC CAC TCG GCC ATC TCT CCTA			
trnS	CGA GGG TTC GAA TCC CTC TC	Demesure 1995	57,5	94º(4')/92º(45")/Tan(45")/72º(3')/72º(10')
trnT	AGA GCA TCG CAT TTG TAA TG			
trnH(GUG)	ACT GCC TTG ATC CAC TTG GC	Hamilton 1999	53	96º(5')/96º(45")/Tan(1')/72º(30")/72º(5')
psbA	CGA AGC TCC ATC TAC AAA TGG			
trnS(GCU)	GCC GCT TTA GTC CAC TCA GC	Hamilton 1999	52	96º(5')/96º(45")/Tan(1')/72º(30")/72º(5')
trnG(UCC)	GAA CGA ATC ACA CTT TTA CCA C			
rpl20	TTT GTT CTA CGT CTC CGA GC	Hamilton 1999	53	96º(5')/96º(45")/Tan(1')/72º(30")/72º(5')
5'-rps12	GTC GAG GAA CAT GTA CTA GG			
trnT(UGU)	CAT TAC AAA TGC GAT GCT CT	Taberlet, 1991	50-55	94º(1')/Tan(1')/72º(2')
trnL(UAA)	TCT ACC GAT TTC GCA TAT C			
trnL(UAA)	GGT TCA AGT CCC TCT ATC CC	Taberlet, 1991	52	95º(1')/95º(15")/52º(15")/72º(10")/72º(1')
trnF	ATT TGA ACT GGT GAC ACG AG			

a total of 30 independent runs with10 million generations were carried out, sampling every 10000th generation. Tracer 1.6 (Rambaut et al., 2014) was used to check convergence of the model and parameters between each run. Following Viruel et al. (2016) clades with bootstrap percentages (BP) of 75-100 or posterior probabilities (PPS) of 0.95-1.0 were considered moderately to strongly supported. The resulting tree files were combined (each independent run with the first 10% samples as burn-in) using LogCombiner 1.8.4 (Drummond et al., 2012). Post-burnin samples were combined across runs to summarize parameter estimates and used to construct a Maximum Clade Credibility tree with median node heights in TreeAnnotator 1.8.4 (Drummond et al., 2012). The phylogenetic reconstructions were calculated for plastid and nuclear markers independently, and for a combined dataset including the three plastid datasets.

In order to test for significant conflicts between the independent DNA data matrices, a partition homogeneity test was performed (Farris et al., 1994, Symonds & Lloyd, 2003). The Incongruence Length Difference (ILD) test of Farris et al. (1994) implemented in PAUP ver.4.0b 10 (Swofford, 2003) was performed through 1000 random-order-entry replicates to estimate if the nuclear and the three plastid data sets were significantly different from random partitions of the same size. Significant results indicate data sets were heterogeneous.

Molecular clock was constructed using BEAST v1.10.3 (Drummond et al., 2012). Because *Lysimachia* belongs to a clade with a poor fossil record, the clock was calibrated using the date estimated by Yesson et al. (2009) for *Lysimachia arvensis* (20 Mya). We choose uncorrelated relaxed clock, using a Yule process, a substitution model TN93, and four gamma categories. Analyses were run for 10 million generations (sampling every 1000th), with a burn-in of one million generations.

Recombination Analysis

To test possible recombination events in the ITS sequences of all the samples, the seven methods (RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan & 3SEQ) implemented in RDP4 v.484 were applied to determinate parental and recombinant sequences with their probability scores for hypothetical recombination events (Martin et al., 2010; Viruel et al., 2018).

RESULTS

Phylogenetic reconstructions and chronogram inference

All the 37 (pDNA)/56(ITS) analysed samples were successfully sequenced for the used markers. The plastid marker fragments were 675-750 bp long in *rps16–trnK*, 580-630 bp for *rpl32-trnL* and 365-445 bp for trnH-psbA. The ITS1 and ITS2 fragments ranged from 600 to 737 bp. The G+C content in all the samples was variable and the means in each marker was 22.22% for *rps16–trnK*, 27.25% for *rpl32–trnL,* 29.04% for *trnH-psbA* and 57.88% for ITS. The G+C content were significantly different between *L. arvensis* and *L. monelli* for ITS (58.86% and 56.46%, respectively) and for rps16-trnK (22.17% and 22.30% respectively). No significant differences were found on the G+C content among blue and red individuals of *L. arvensis* (p=0.482) or *L. monelli* (p=0.338).

The parsimony, RAxML and Bayesian trees displayed the same topology as much in ITS as consensus pDNA markers. The tree obtained with the three plastid markers showed the same topology. However, the ILD test showed significant differences between nuclear and plastid regions, indicating that there was an inconsistency between these markers. Thus, phylogenetic analyses were performed for the ITS region separately from the concatenated dataset representing the three plastid markers (p=0.010).

The ITS maximum clade credibility (MCC) tree showed two main clades (Fig. 2). In the Clade I (98 BS, 1.000 PPS) blue *L. arvensis* samples (89 BS, 1.000 PPS) are brothers to *L. talaverae* (95 BS, 0.999 PPS), where the samples of *L. arvensis* from SW of Spain form a group (82BS, 1.000 PPS). The Clade II (87 BS, 1.000 PPS) comprised two subclades: A and B. In the subclade A (85 BS, 0.955), red *L. arvensis* samples (84BS, 0.976 PPS) are brothers to red *L. monelli* samples (85BS, 1.000 PPS). In the subclade B (89BS, 0.999), blue *L. monelli* samples (83 BS, 0.984 PPS) are brothers to *L. foemina* (95BS, 1.000 PPS). Therefore, blue and red individuals of both *L. arvensis* and *L. monelli* appeared into different groups. It is noteworthy that blue and red individuals from mixed populations of *L. arvensis* appeared in independent clades.

According to the relaxed molecular clock, the common ancestor of blue plants of *Lysimachia arvensis* and *L. talaverae* diverged around 11 Mya (Fig. 2, Clade I) and the common ancestor of the Clade II around 10 Mya. The divergence of blue plants of *L. monelli* and *L. foemina* around 9.5 Mya and the split between red plants of *L. arvensis* and red plants of *L. monelli* is dated around 8.5 Mya.

The consensus pDNA ML tree showed two clades (Fig. 3). The first clade (94 BS, 0. 997 PPS) comprised all the red and some blue individuals of *L. arvensis* and *L.*

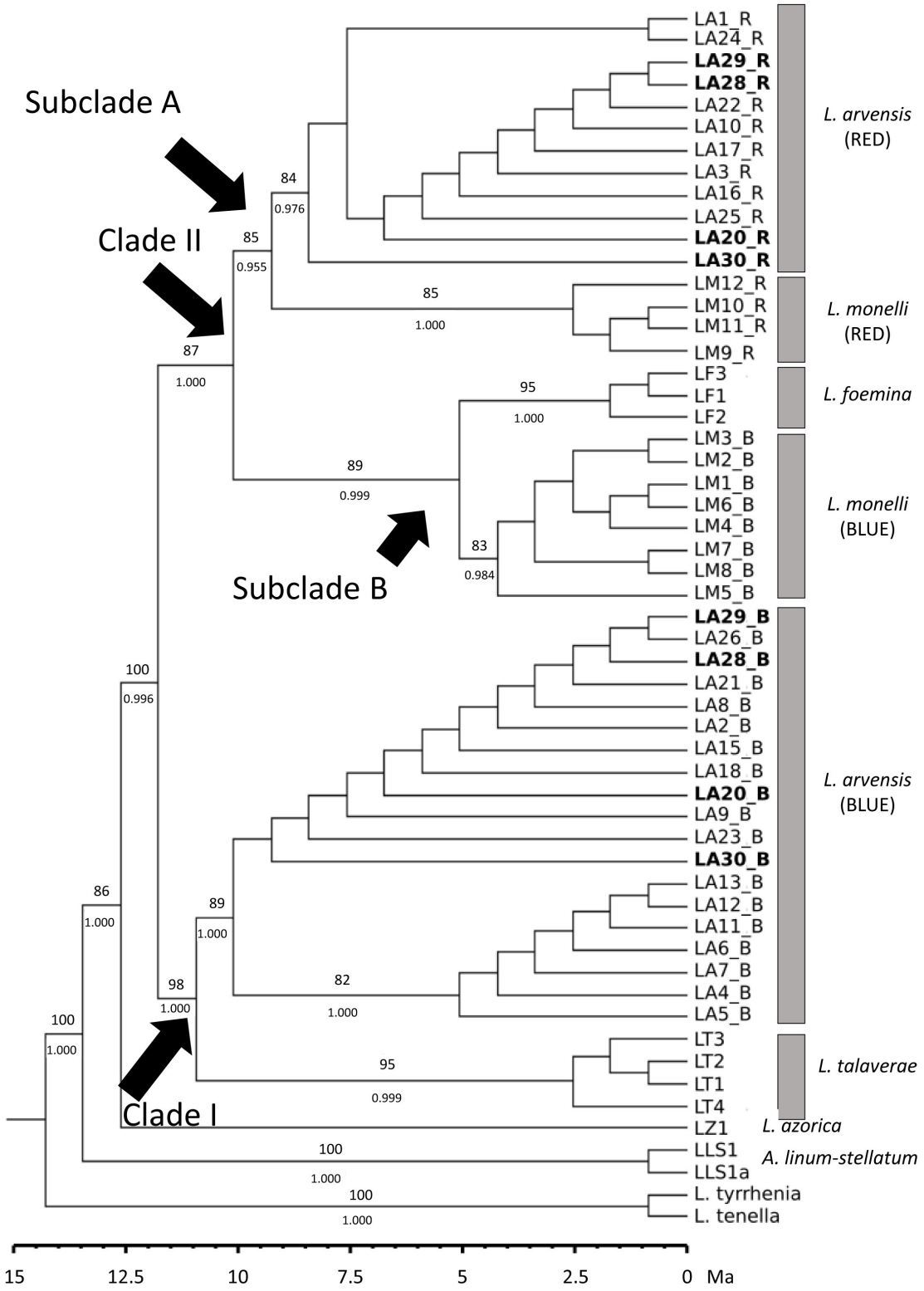

Figure 2. MCC trees based on ITS sequences of *L. arvensis* and *L. monelli* and related taxa (see Table 1). Bootstrap values > 50% and posterior probabilities of Bayesian tree are given above and below the branches respectively. The samples of *L. arvensis* from mixed populations highlighted in bold, when appear in different clades.

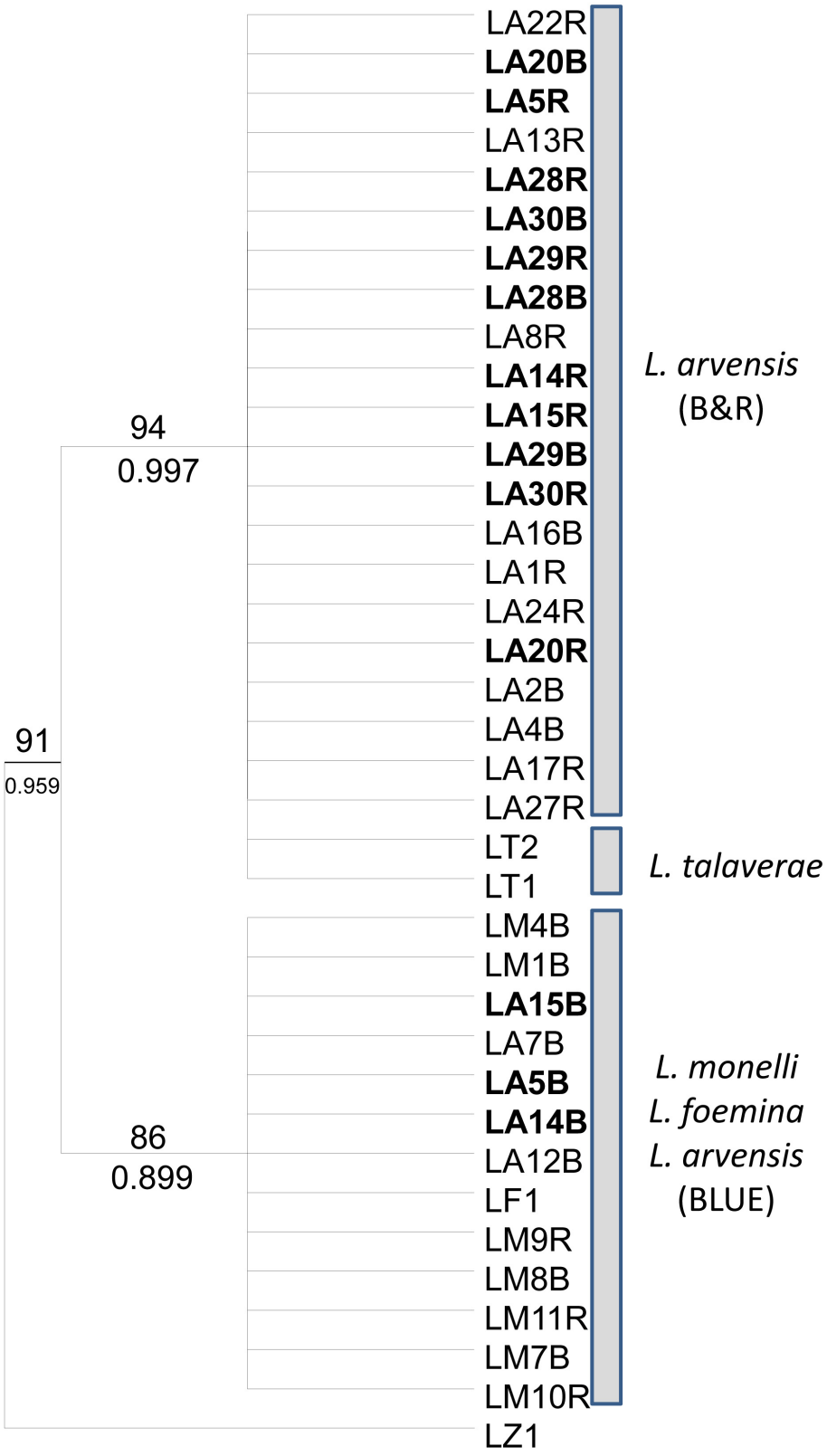

Figure 3. Consensus ML tree based on *trnH–psbA*, *rps16– trnK* and *rpl32-trnL* sequences of *L. arvensis* and *L. monelli*, and related taxa (see Table 1). Bootstrap values > 50% and posterior probabilities of Bayesian tree are given above and below the branches respectively. The samples of *L. arvensis* from mixed populations highlighted in bold, when appear in different clades

talaverae. The second clade (86 BS, 0.899) included blue individuals of *L*. *arvensis*, *L*. *monelli* (blue and red) and *L*. *foemina*. The independently reconstructions of rps16-trnK and *rpl32-trnL* showed a very similar tree topology than that of the consensus pDNA tree.

Recombination Analyses

In the phylogenetic reconstruction with nuclear marker, four of the seven applied methods detected two recombinant regions in the sample LA19_B. In that sample, an insertion of 18 nucleotides at position 16 from *L*. *arvensis* and an insertion of 25 nucleotides at position 133 from *L*. *foemina* were observed. That sample was discarded from the tree in figure 2. The tree with LA19_B is shown in the appendix 1.

DISCUSSION

Colour morphs of *L. arvensis* and *L. monelli* appeared together with all the plastid markers studied. A general congruence was found between analyses of the three pDNA markers despite few informative sites per region. This result was expected because the plastids are inherited as haplotype (Wolfe et al., 1987). The pDNA haplotype shared among closely related species is common for many different plant taxa (Wolf et al., 1997; Matsumura et al., 2009). However, colour morphs of *L. arvensis* appeared separated with the nuclear marker. The presence of different ITS sequences in the red and blue flowered individuals of *L. arvensis* collected in the same locality had already been reported by Manns & Anderberg (2007a) but they did not explain it and suggested the necessity of further studies.

Although the two colour morphs of *L. monelli* currently are recognized under the same species (Ball, 1878; Battandier & Trabut, 1905; Pujadas, 1997), also had different ITS sequences and are in different subclades. Samples of *L. monelli* with red flowers are in Subclade A (85 BS, 0.955 PPS), very related with all samples of *L. arvensis* with red flowers. However, *L. monelli* with blue flowers are in Subclade B (89 BS, 0. 999 PPS) with *A. foemina*. That support that *L. foemina* is an independent species to *L. arvensis*, in accordance with the results of Manns & Anderberg (2007a). Therefore, the two colour morphs of *L. monelli* cannot belong to the same species. With a superior support to 75BS or 0.95PPS we can consider significant enough to define different species (Viruel et al., 2016). Them, the support of our ITS results (87 BS, 1.000 PPS) have to consider blue and red samples of *L*. *monelli* as different species.

The blue morph of *L. monelli* was described as *Anagallis monelli* by Linnaeus (1753) of Cádiz (Spain). This species is very frequent in the W, C and S of Iberian Peninsula and very rare in the NW of Africa. It presents a great morphological variability in the shape

of the leaves, length of the pedicels, size of flowers and capsules, for which several taxa have been described (Linnaeus, 1762; Mariz, 1899-1900; Sampaio, 1900; Coutinho, 1939; Sennen, 1936). In our study, 8 populations were studied, 6 from the Atlantic coast from Cádiz (Spain) to Estremadura (Portugal) and 2 from Castilla-León (Salamanca and Zamora), which largely represent all the variation described, but our ITS results show nothing that justifies any of the described taxa and we have considered here to be mere synonyms for *L. monelli* s. str.

The red morph of *L. monelli*, as indicated in the introduction, was first described by Schousboe (1800) as *Anagallis collina* Schousb., from Mogador region (Morocco). This species is very similar morphologically to *A. monelli* with which many authors have synonimized it, but it differs from the latter, by having the stems much more woody and branched, oval-lanceolate leaves, always crass and shorter than the internodes, red or pink flowers, generally greater than those of *A. monelli*. *Anagallis collina* is very frequent in Morocco, especially in the littoral or sublittoral where it coexists with *Argania spinosa*, *Tetraclinis articulata* and *Juniperus phoenicea*, but also in the Atlas Mountains, especially in the calcareous scree of *Quercus rotundifolia*. It is less frequent in Algeria, rare in Tunisia and very rare in Sardinia and NE of Spain, especially on the coast of Tarragona and Castellón, where it is in serious danger of extinction. From Tarragona, Willkomm (1870) described *Anagallis collina* var. *hispanica* Willk. and also, from Tarragona, Sennen (1936) described *Anagallis willkommii* Sennen. In this work we have studied 4 populations: two from Morocco, one from Sardinia and one from Tarragona. Our ITS results indicate that nothing justifies the separation of Spanish plants from other populations.

Moreover, our ITS results indicated that blue individuals of *L. arvensis* are sister to *Lysimachia talaverae* (Fig.2, Clade I). The blue morph of *Lysimachia arvensis* was described by Linnaeus (1753) as *Anagallis latifolia* L., but many authors have confused it with *Anagallis arvensis* var. *caerulea* sensu (Kollmann & Feinbrun, 1968; Pujadas, 1997). On the other hand, the red morph of *Lysimachia arvensis*, described by Linnaeus (1753) as *Anagallis arvensis* L, is part of the Subclade A where it is brother of the red morph of *L. monelli*; therefore both morphs of *A. arvensis* belong to different taxa.

Both morphs are very similar but blue morph differs for being more robust plants, leaves bigger, often acute, cordiform at the base and flowers up to 15 mm in diameter in the anthesis. Blue morph also resembles his phylogenetic brother, *Lysimachia talaverae*, but the latter species is smaller, with secondary roots in the root neck, flowers 2-4 (5) mm in diameter in the anthesis, usually of a pale blue and smaller anthers and style. These three species have in common that the margin of the petals

present numerous tricellular glands with the globose terminal cell and much larger than the adjacent cell. Moreover, blue morph differs from *L. foemina*, with which it shares the blue colour of the flowers, since the latter has the radical neck, as in *L. talaverae*, covered with secondary roots, lanceolate and sharp leaves, especially those of the upper half of the stem, pedicel shorter and thicker in fruit, smaller flowers, up to 8 mm in diameter in anthesis and the corolla lobes cuneate, dentate, glabrous, but more often with some hairs in the margin formed by (3) 4 (5) cells, all more or less cylindrical. Therefore, these 4 annual taxa differ in morphological and phylogenetic relationships, so they should be considered with taxonomic category of species. All of them are self-compatible with short-lived flowers (2-3 days), most of them self-pollinated when the anthers touch the stigma at the end of the first day of the flower (Gibbs & Talavera, 2001), so that cross pollination is rarely produced by very small solitary bees, as was demonstrated in several populations of England where *L. arvensis* and *L. foemina* coexisted (Marsden-Jones & Weiss, 1960). This quality together with the fact that blue and red *L. arvensis* and *L. foemina* are tetraploids means that, although they are frequent in the cultivated fields of the Mediterranean region, they have colonized other regions of the Mediterranean climate of the World, possibly propitiated by the transfer of cereal seeds to those regions. On the contrary, *L. talaverae* is diploid and lives in humid depressions so, despite being self-compatible and autogamous (Gibbs & Talavera, 2001), it only colonizes habitat of the W of Mediterranean region, mainly in the Atlantic littoral of the Iberian Peninsula, probably intervening in the dispersion of seeds the aquatic birds by epi-zoocoria so frequent in these ecosystems. This last species has already been recognized with the rank of species based on morphological, ecological and cariological characters but not on molecular tools and so, our study corroborates the separation of *L. talaverae* as species.

It is note that in each clade or subclade there is a couple of diploid and tetraploid taxon. We could hypothesize a common ancestor with 2n=20 or 40 chromosomes that up to three times doubled or reduced its genetic material. The molecular clock does not clarify if the common ancestor was diploid or tetraploid but a doubled of genetic material is the most likely scenario. Šveřepová (1978) has already proposed that tetraploids originated from diploids by hybridization. It is also remarkable that two of the tree couples have a taxon annual and self-compatible and other taxon perennial and self-incompatible. Transitions between annual and perennial habit in both directions can be quite frequent among herbaceous species (Barrett et al., 1996; Talavera et al. 2011). This suggests considerable lability of life cycle.

Taking into account the results obtained with both markers, we observed differences among the plastid consensus tree and ITS tree. These differences may be explained for the comparatively lower rate of evolution of plastid markers vs nuclear markers

(Wolfe et al., 1987), even assuming that individuals from the same population are similar for phylogenetic studies (Degnan & Rosenberg, 2009). Hybridization has been proposed as causes of incongruences between pDNA and nuclear phylogenies (Wendell & Doyle, 1998; Semerikova & Semerikov, 2016). Hybridization involves contact or hybrid zones (Petit et al., 1999) where populations with divergent genomes have the potential to exchange genes (Souissi et al., 2017). But as a result of this exchange we would not expect to find the clear separation between colour morphs observed in the phylogenetic reconstruction with nuclear markers (Fig. 2). Otherwise, the coalescent theory (Nordborg, 2001; Wakeley, 2009) may explain the branching patterns of phylogenetic trees (Degnan & Rosenberg, 2009), because two or more lineages can coexist in the same ancestral population (Degnan & Rosenberg, 2009). In our study only one sample presents signal of hybridization (LA19B; appendix 1). So, the common ancestor of this group would already have the two pDNA haplotypes present in the current species: one of them in red individuals of *L. arvensis* and *L. talaverae*, the other in *L. monelli* and *L. foemina*, and both haplotypes in blue individuals of *L. arvensis*. Later, a segregation of colour morphs of *L. arvensis* and *L. monelli* happened. That segregation could have been promoted by geographic separation, assortative mating mediated by pollinators or by differential tolerance to abiotic factors as has been described in other species (Kirkpatrick, 2000; Strauss & Whittal, 2006; Hopkins & Rausher, 2012; Wang et al., 2013). In *L. monelli* geographic separation could be contributing to morph separation. However, in *L. arvensis* genetic flow is potentially possible between morphs in polymorphic populations, but the results of nuclear phylogenetic reconstruction points towards a clear isolation of both morphs.

The position of the sample LA19B in the ITS tree would suggest a possible hybridization between blue *L. arvensis* and *L. foemina*. The two species co-occur in many populations in Europe and all the floral traits are very similar with the difference in the shape of the petals and the number of cells in the glands of the petal margin (Marsden-Jones & Weiss, 1938; Manns & Anderberg, 2007a), thus they possibly share pollinators. Unexpectedly, the recombination analysis confirms the hypothesis of hybridization between red *L. arvensis* and *L. foemina.* Thus, that cross results in an individual with blue flowers as *A. foemina* but bigger, similar to the phenotype of the blue *L. arvensis* (as was initially considered in the recollection). This fact is interesting as a similar phenotype seems to be obtained by the crossing of two taxa that appear so far apart in phylogenetic reconstruction. These hybrids (*L. arvensis* x *L. foemina*) have already been described as *A.* x *doerfleri* Ronniger (Dörfler, 1903), but crosses between these two species resulted in sterile F1 progeny (Marsden-Jones, 1935), since the pollen is atrophied (Kollmann & Feinbrun, 1968; Šveřepová, 1972).

Flower colour constitutes a pivotal evolutionary force to speciation in several groups of plants (Carlson & Holsinger, 2015; Ellis & Field, 2016; Takahashi et al., 2016, Narbona et al. 2018). Our study demonstrates the delimitation of independent taxa within *Lysimachia arvensis* and *L. monelli* based on phylogenetic inference and concomitant with flower colour. Although both species show differentiation by flower colour, their geographical patterns are very different. In *L. arvensis* we found some mixed populations whereas in *L. monelli* only pure populations have been sampled. Species as *Ipomoea purpurea*/ *I. nil* (Miller et al., 1999; Clegg & Durbin, 2000) or *Primula vulgaris*/ *P. veris* (Volkova et al., 2013) showed a situation similar to *L. monelli*, where the geographical separation maintains the entity of taxa. On the other hand, in *Iris lutescens* (Wang et al., 2016), *Nigella degenii* (Jorgensen et al., 2005) or *Limonium wrightii* (Matsumura et al., 2006) there were mixed populations, as in *L. arvensis*, and the molecular analysis was not enough to establish the category of species to the different morphs due to evidence of gene flow between morphotypes. However, in *Schlumbergera* or *Hatiora* genera, the support of the phylogenetic reconstruction allowed different morphotypes of colour to be described as different species (Calvente et al., 2011). With our results we consider *L. arvensis* is in the last situation because we have evidence of limited gene flow between morphs (chapter 7).

In conclusion, the two colour morphs of *L. arvensis* constitute at present independent clusters and their current isolation are evident, blue morph of *L. arvensis* is a taxon more adapted to the arid conditions of the Mediterranean while red morph is a more mesic taxon, with a greater distribution area and that coexists with blue morph in some areas in the Mediterranean climate. In the case of *L. monelli*, in the absence of mixed populations found, and although both colour morphs seem to have the same origin, they are also independent entities. Therefore, a phylogeographic approach is necessary to clarify the geographical distribution of both colour morphs. Consequently, our results have taxonomic implications and morphs of *L. arvensis* and *L. monelli* should be defined as different species, with independent morphological, genetic and geographic identity.

Taxonomic implications

The results obtained with the ITS markers have taxonomic implications for both colour morphs of *L. arvensis* and *L. monelli*.

-Red plants of *Lysimachia arvensis* should maintain this name since Linnaeus in 1753 used plants with red flowers to describe the species.

Lysimachia arvensis (L.) U. Manns & Anderb. in Willdenowia, 39(1):51. (2009) ≡ *Anagallis arvensis* L., Sp. Pl.: 148. 1753, [basion.] ≡ *Anagallis arvensis* var.

phoenicea Gouan, Fl. Mons.: 24 (1764), nom. Illeg. ≡ *Anagallis phoenicea* (Gouan) Scop., Fl. Carn. ed. 2, 1: 139 (1777), nom. Illeg. ≡ *Anagallis arvensis* subsp. *phoenicea* (Gouan) Wallmann in Ber. Bayer. Bot Ges. 9: 44 (1904), nom. inv.
Ind. Loc.: "Habitat in Europae arvis".

Lectotype designated by Dyer & al. 1963: 14: Herb. Linn. 208.1 (LINN)

-Blue plants of *Lysimachia arvensis* should be called with the specific epithet latifolia due to it was the first name employed by Linnaeus in 1753 for plants with blue flowers. However, the epithet latifolia already exists within *Lysimachia* to refer to a different taxa [*Lysimachia latifolia* (Hook.) Cholewa in Phytoneuron 28: 1-2 (2014)]. Therefore we select the name *Lysimachia loeflingii* because Linnaeus in 1753 described these plants from materials collected by Loefling from Spain.

Lysimachia loeflingii FJ. Jiménez-López & M. Talavera, **nom. nov.** ≡ *Anagallis latifolia* L., Sp. Pl. 1: 149 (1753) [syn. subst.] ≡ *Anagallis arvensis* subsp. *latifolia* (L.) Arcang., Comp. Fl. Ital., ed.2: 456 (1894) = *Anagallis arvensis* L var. *caerulea* sensu Kollmann & Feinbrun in Notes Royal Bot. Gard. Edingurg 27: 176 (1968), non *Anagallis arvensis* L. var. *caerulea* (L.) Gouan, Fl. Monsp.: 30 (1765)
Ind. loc.: "Habitat in Hispania Loef.".

Lectotype designated here: Herb. Linn. nº 208.3, "H.U.3. latifolia. ex Hispanica foly amplexicauly" [m. Linnaeus]

Anagallis latifolia L. was described by Linnaeus in the 1st edition of his Species Plantarum (1753: 149) indicating: "3. Anagallis foliis cordatis amplexicaulibus, caulibus compressis latifolia/ Anagallis hispanica, latifolia, maximo flore. Tournef. Inst. 142/ Cruciata Montana minor, flore caeruleo. Barr. Ic. 584/ Habitat in Hispania Loefl. "

Then he gives a very detailed description of the plant, except for the fruit, indicating also the colour of the flowers, "corolla caerulea, fondo purpurascens," and of the stamens, "Filamenta purpurea. Antheris oblongis, flavis. "

The reference made by Linnaeus to Tournefort (1719: 142) corresponds, according to Sampaio (1900: 57), to a plant collected by Tournefort in "Ultra San Joan de Foz ad ostium durii" on his travel through Portugal in 1689, as it appears in a manuscript that Tournefort left in the Museu Botanico da Universidade (Coimbra). This name phrase of Tournefort was used by Sampaio (1900: 58) as a synonym of *Anagallis hispanica* Sampaio, which we consider here synonymous of *Anagallis monelli* L. (see below this species).

The icon 584, of the Lam. 157, by Barrelier (1714: 17) indicated by Linnaeus very faithfully represents the upper part of a branch with large flowers and with all the

lanceolate leaves arranged in whorls of 4 in each node, each with its flower. This plant can also be identified with *Anagallis monelli* L. Therefore, in none of the synonyms that Linnaeus gives in *Anagallis* latifolia could the type be chosen since both the Tournefort plant and the Barrelier icon have characters very different from those described by Linnaeus in *Anagallis latifolia*. Therefore the type would have to choose it among the material of herbarium that Loefling sent to his Master from Spain, as Linnaeus indicated in the locotypical indication.

In the different Linnaean herbariums we do not find any material of *Anagallis latifolia* collected by Loefling in Spain, but what is found in the main herbarium of Linnaeus (LNN) is the sheet No. 208.3, with the indication, handwritten by Linnaeus "HU" at the base of the plant, and at the base of the sheet, also with Linnaeus's letter, "3 latifolia". 3 is the order that Linnaeus gave to his *Anagallis latifolia* when he described it, that is, we are dealing with a material that comes from the crops of the Botanical Garden of Upsala and that was marked by Linnaeus as material of the first edition of his Species Plantarum of 1753, and therefore the material could be the type of the species. In addition, in an attached cut-out to the sheet and handwritten by Linnaeus "*Ex Hispanica foly amplexicauly*" is indicated. With this statement Linnaeus relates the cultivated plant in *Hortus Upsaliensis* with the one from Spain that Loefling sent him. The material that is in the sheet consists of the upper half of a flowered plant of 20 cm; quadrangular stems, with internodes 2-5 cm; opposite leaves, those greater 3 x 2 cm, broadly ovate-lanceolate, sharp, cordate at the base; solitary, axillary flowers, with capillary pedicels of 20-30 mm, almost the length of the leaves; blue corolla, with petals of c. 7 mm in length, and immature capsules much smaller than the calyx. It is evident that this material comes from cultivation, as indicated Linnaeus. But also it is that Linnaeus made the detailed description of the species on the living plant, since some characters, especially those that refer to the two colours of the corolla, and the colour of the filaments and the anthers of the stamens, he could not describe them with the pressed material like the one found in Linnaeus' main herbal sheet. This material from the Upsala Garden came from the seeds that Loefling sent to Linnaeus. For all the above, we propose as lectotype of *Anagallis latifolia* L. the only plant found in Linnaeus' main herbarium (LINN) nº 308.3.

-The plants with very small blue flowers that live in the environment of the wetlands have been included sometimes in *Anagallis arvensis* so a typification proposal is made.

Lysimachia talaverae L. Sáez & Aymerich in Orsis 29: 48 (2015) ≡ *Anagallis parviflora* Hoffmanns. & Link, Fl. Portug. 1: 325, Tab. 64 (1813-1820) [syn. subst.] ≡ *Anagallis arvensis* subsp. *parviflora* (Hoffmanns. & Link) Arcangeli, Comp. Fl. Ital. ed.2: 456

(1894) = *Anagallis latifolia* “raza” *parviflora* (Hoffmanns. & Link) Merino in Broteria , sér. Bot. 14: 162 (1916), nom. illeg.
Ind. loc.: “Dans les lieux sablonneux aux environs de Comporta”.

Lectotype designated here (iconotype): *Anagallis parviflora* Tab. 64 in Hoffmannsegg & Link, Fl. Portug. 1: 326 (1813-1620).

Epitype: Huelva. Hinojos, Las Porqueras. Lagoon edge. 37º 17' 37" N-6º 25' 15"W. 108 m. 5/5/2014. Leg. F.J. Jiménez & S. Talavera. SEV286467. A sheet of this collection has been analyzed for phylogenetic work with a nuclear marker (ITS) and three chloroplastic markers.

-*Lysimachia monelli* should be referred exclusively to the blue morph because it was used by Linnaeus in 1753 to describe this species for the first time.

Lysimachia monelli (L.) U. Manns & Anderb. in Willdenowia, 39(1): 52 (2009) ≡ *Anagallis monelli* L., Sp. Pl. 1: 148 (1753), [basion.].

Ind. Loc.: not indicated by Linnaeus in 1753; "Habitat in Verona" in Linnaeus, Sp. Pl. ed. 2, 1: 212. 62; As indicated Carlos Pau (1915) the locotypical indication was clearly exposed by Linnaeus when he described the species when indicating his previous synonym, both in his Hortus cliffortianus, 52. 1738: "Crecit forte Gadibus und seminis accepit Joh. Monellus Tornacensis, atque eaden cum Clussius comunicavit anno 1602 ", as in Hortus upsaliensis, 38. 1748: “Habitario incerta. Johannes Monellus 1662 [1602] Gadibus semina Clussius misit. Hospitatur in Terpidario, perennes”.

Lectotype designated by Manns & Anderberg in Willdenowia 39 (1): 52 (2009): Hort. Cliff. 52.2, Anagallis 2 (BM000557969).

The lectotype is formed by two flowering stems of c. 30 cm, one undivided and the other branched almost from the base, each with numerous flowers and only those of the apex in anthesis, the others in postantesis; internodes 2-5 cm, greater than leaves; opposite inferior leaves, of 20-25 x 5 mm, elliptical, attenuate in the base, acute in the apex, superior in whorls of 3 leaves of 15-20 x 3-5 mm, elliptical, each one with a long pedicelled flower; pedicels of 25-35 mm, generally larger than the leaves. These branches come from the garden of George Clifford III (Hartekamp Garden, Holland) that should never have been exposed to pollinators since none of the numerous post-anthesis flowers developed fruit.

= *Anagallis linifolia* L. Sp. Pl. ed. 2, 1: 212 (1762)

≡ *Anagallis monelli* subsp. *linifolia* (L.) Maire in Jahandiez & Maire, Cat. Pl. Moroc.: 562 (1934)

Ind. Loc.: “Habitat in Lusitania, Hispania. Claud Alstroemer”.

Lectotype designated here: “linifolia [m. Linnaeus] A [Alstroemer]”: Herb. Linn. Nº 208.4 (LINN-ML)

The lectotype is formed by a single apparently annual plant of 10 cm high, erect, with 3 short branches in fruit in the upper half and some branches emerging in the lower, near of the axonomorph root; stem with very short internodes, surpassed by the leaves; leaves of 6-10 x 0.1-0.2. mm, narrowly linear, truncated at the base, obtuse; pedicels all with fruit, recurved, rigid; fruits shorter than the calyx. The characters of this plant coincide with those indicated by Linnaeus when he described the species, so there is no doubt that this plant is the same as Linnaeus saw. The indication of Lusitania should have been taken by Linnaeus from a plant in Portugal described by Tournefort and cited as a synonym (*Anagallis lusitanica*, linariae folio angustiore, Tournefort 1719 pag. 143).

This type of apparently annual plants such as the type are very frequent on the coast of the province of Cádiz near the capital, so it is not surprising that Linnaeus thought when he published *A. linifolia* that had this type of life cycle. According to Fernández Pérez (1990), Class Alstroemer toured these territories in the spring of 1760, so the type probably comes from this region of Cádiz.

-Red plants of *Lysimachia monelli* should be called *Lysimachia collina* because plants with this colour were described by Schousboe in 1800 as *Anagallis collina*. Due to *Anagallis* now are included in *Lysimachia*, we do a new combination.

Lysimachia collina (Schousb.) FJ. Jiménez-López, **com.nov.** ≡ *Anagallis collina* Schousb., Iagttg. Vextrig. Marokko.: 78. 1800, basion. ≡ *Anagallis monelli* subsp. *collina* (Schousb.) H. Lindb. in Acta Soc. Sci. Fenn., ser. B, Opera Biol. 1(2): 115. 1932.

Ind. loc.: “Frequens ocurrit in collibus aridis provinciae Haha.”.

Lectotype designated here: Mogador [Haha] Schousboe [m. Schousboe]: C 10001180.

The type material is formed by a single very woody stem of 15 cm in length, without leaves in the lower half, branched in the upper half; each branch short, with some oval-lanceolate leaves, acute, shorter than the pedicels and several flowers, the majority in anthesis.

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REFERENCES

Anderberg AA, Manns U & Källersjö M. (2007). Phylogeny and floral evolution of the Lysimachieae (Ericales, Myrsinaceae). Evidence from ndhF sequence data. *Willdenowia*, **37**: 407–421.

Arcangeli G. (1894). Compendio della Flora Italiana **2**: 456. Torino and Roma.

Arista M, Talavera M, Berjano R & Ortiz PL. (2013). Abiotic factors may explain the geographical distribution of flower colour morphs and the maintenance of colour polymorphism in the scarlet pimpernel. *Journal of Ecology*, **101(6)**: 1613–1622.

Aymerich P & Sáez Ll. (2015). Comentaris i precisions previs a la Checklist de la flora de Catalunya (Nord-Est de la península Ibèrica). *Orsis*, **29**: 23–90.

Ball J. (1878). Spicilegium florae maroccanae. Part III. *The Journal of the Linnean Society, Botany* **16 (25)**: 472–568.

Barrelier J. (1714). Plantae per Galliam, Hispaniam et Italiam. Ed. Antoine de Joussieu. Parisii.

Barrett SCH, Harder LD & Worley A. (1996). The comparative biology of pollination and mating in flowering plants. *Philosophical Transactions of the Royal Society of London, B, Biological Sciences* **351**: 1271–1280.

Battandier A, Trabut L. (1905). Notes sur quelques plantes de la flore atlantique. *Bulletin de la Société Botanique de France* **52**: 498–502.

Bolòs O & Vigo J. (1996). Flora dels Països Catalans. 3. Ed. Barcino. Barcelona.

Calvente A, Zappi DC, Forest F & Lohmann LG. (2011). Molecular phylogeny of tribe Rhipsalideae (Cactaceae) and taxonomic implications for *Schlumbergera* and *Hatiora*. *Molecular Phylogenetics and Evolution*, **58(3)**: 456–468.

Carlson JE & Holsinger KE. (2015) Extrapolating from local ecological processes to genus-wide patterns in colour polymorphism in South African Protea. *Proceedings of the Royal Society of London B: Biological Sciences*, **282**: 20150583.

Chase MW & Hills HH. (1991). Silica gel: an ideal material for field preservation of leaf samples for DNA studies. *Taxon*, **40 (2)**: 215–220.

Clegg MT & Durbin ML. (2000). Flower color variation: a model for the experimental study of evolution. *Proceedings of the National Academy of Sciences*, **97(13)**: 7016–7023.

Coutinho AXP. (1939). Flora de Portugal ed.2. Publ. Bertrand (Irmaos), Ltd. Lisboa

Darriba D, Taboada GL, Doallo R & Posada D. (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods,* **9(8)**: 772.

Degnan JH & Rosenberg NA. (2009). Gene tree discordance, phylogenetic inference and the multispecies coalescent. *Trends in Ecology & Evolution*, **24(6)**: 332–340.

Dörfler I. (1903). Herbarium Normale XLV: 143.

Drummond AJ, Suchard MA, Xie D & Rambaut A. (2012). Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution,* **29**: 1969–1973.

Dyer RA. (1963). Primulaceae. In Dyer RA, Codd IE & Rycroft HB. (eds.). Flora of Southern Africa. Pp: 9–14. Pretoria.

Ellis TJ & Field DL. (2016) Repeated gains in yellow and anthocyanin pigmentation in flower colour transitions in the Antirrhineae. *Annals of Botany*, **117**: 1133–1140.

Farris JS, Kellersjo M, Kluge AG & Bult C. (1994). Testing significance of congruence. *Cladistics,* **10**: 315–320.

Ferguson LF. (1972) *Anagallis* L. In Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM & Webb DA. (eds.). Flora Europea 3, Diapensiaceae-Myoporaceae. Pp. 28–29. Cambridge University Press. Great Britain.

Fernández Pérez J. (1990). Tres apóstoles de Linné en Cádiz: Pehr Osbeck, Pehr Löfling y Clas Alströmer. In Pelayo López F (ed.). Pehr Löfling y la expedición al Orinoco. Pp.51–102. Sociedad Estatal V Centenario. Madrid.

Freyre R & Griesbach RJ. (2004). Inheritance of flower color in *Anagallis monelli* L. *HortScience*, **39(6)**, 1220–1223.

Gibbs PE & Talavera S. (2001). Breeding system studies in three species of *Anagallis* (Primulaceae): self-incompatibility and reduced female fertility in *A. monelli* L. *Annals of Botany*, **88**: 139–144.

Hamilton MB. (1999). Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. *Molecular Ecology*, **8(3)**: 521–523.

Hao G, Yuan YM, Hu CH, Ge CH & Zhao NX. (2004). Molecular phylogeny of *Lysimachia* (*Myrsinanceae*) based on chloroplast *trnL-F* and nuclear ribosomal ITS sequences. *Molecular Phylogenetics and Evolution*, **31**: 323–339.

Harborne JB. (1968). Comparative biochemisty of the flavonoids – VII. Correlations between flavonoid pigmentation and systematics in the family Primulaceae. *Phytochemistry*, **7**: 1215–1230.

Hoffmannsegg JCG & Link JHF. (1813-1820). Flore Portugaise, 1: 325, Tab. 64. Impr Charles Fréderic Amelang. Berlin.

Hopkins R & Rausher MD. (2012). Pollinator-mediated selection on flower color allele drives reinforcement. *Science*, **335**: 1090–1092.

Jiménez-López FJ, Ortiz MA, Berjano R, Talavera S & Terrab A. (2016). High population genetic substructure in *Hypochaeris leontodontoides* (Asteraceae), an endemic rupicolous species of the Atlas Mountains in NW Africa. *Alpine Botany*, **126**: 73–85.

Jorgensen TH, Richardson DS & Andersson S. (2006). Comparative analyses of population structure in two subspecies of *Nigella degenii*: evidence for diversifying selection on pollen-color dimorphisms. *Evolution*, **60**: 518–528.

Källersjö M, Bergqvist G & Anderberg AA. (2000). Generic realignment in primuloid families of the Ericales s.l.: a phylogenetic analysis based on DNA sequences from three chloroplast genes and morphology. *American Journal of Botany*, **87**: 1324–1341.

Kirkpatrick M. (2000). Reinforcement and divergence under assortative mating. *Proceedings of the Royal Society of London B: Biological Sciences*, **267**: 1649–1655.

Kollmann F & Feinbrun N. (1968). A cyto-taxonomic study in Palestinian *Anagallis arvensis* L. *Notes of the Royal Botanic Garden Edinburgh*, **27**: 173–186.

Kress A. (1969). Zytotaxonomische Untersuchungen an Primulaceen. *Phyton (Austria),* **13 (2-4)**: 211–225.

Linnaeus C. (1753). Species Plantarum **1**: 148-149. Stockholm, Sweden.

Linnaeus C. (1762). Species Plantarum ed. 2, 1. Impres direct laurentii salvii. Olmiae.

Manns U & Anderberg AA. (2005). Molecular phylogeny of *Anagallis* (Myrsinaceae) based on ITS, trn LF, and ndh F sequence data. *International Journal of Plant Sciences,* **166(6)**: 1019–1028.

Manns U & Anderberg AA. (2007a). Relationships of *Anagallis foemina* and *A. arvensis* (Myrsinaceae): new insights inferred from DNA sequence data. *Molecular Phylogenetics and Evolution*, **45(3)**: 971–980.

Manns U & Anderberg AA. (2007b). Character evolution in *Anagallis* (Myrsinaceae) inferred from morphological and molecular data. *Systematic Botany*, **32(1)**: 166–179.

Manns U & Anderberg AA. (2009). New combinations and names in *Lysimachia* (Myrsinaceae) for species of *Anagallis*, *Pelletiera* and *Trientalis*. *Willdenowia*, **39(1)**: 49–54.

Mariz J. (1899-1900). Subsidio para o studio da flora portuguesa. Primulaceae y Gentianaceae. Boletin de la Sociedad Broteriana, **16**: 156–195.

Marsden-Jones EM. (1935). The genetic of *Anagallis arvensis* Linn. and *Anagallis foemina* Mill. *Proceedings of the Linnean Society of London*, **147**: 105–106.

Marsden-Jones EM & Weiss FE. (1938). The essential differences between *Anagallis arvensis* Linn. and *Anagallis foemina* Mill. *Proceedings of the Linnean Society of London*, **150**: 146–155.

Marsden-Jones E. & Weiss FE. (1960). The genetics and pollination of *Anagallis arvensis* subsp. *arvensis* and *Anagallis arvensis* subsp. *foemina*. *Proceedings of the Linnean Society of London*, **171**: 27-29.

Martin DP, Lemey P, Lott M, Moulton V, Posada D & Lefeuvre P. (2010). RDP3: a flexible and fast computer program for analyzing recombination. *Bioinformatics* **26**: 2462–2463.

Martins L, Oberprieler C & Hellwig FH. (2003). A phylogenetic analysis of *Primulaceae* s.l. based on internal transcribed spacer (ITS) DNA sequence data. *Plant Systematics and Evolution*, **237**: 75–85.

Matsumura SI, Yokoyama J, Tateishi Y & Maki M. (2006). Intraspecific variation of flower colour and its distribution within a sea lavender, *Limonium wrightii* (Plumbaginaceae), in the northwestern Pacific Islands. *Journal of Plant Research*, **119(6)**: 625–632.

Matsumura S, Yokoyama J, Fukuda T & Maki M. (2009). Origin of the disjunct distribution of flower colour polymorphism within *Limonium wrightii* (Plumbaginaceae) in the Ryukyu Archipelago. *Biological Journal of the Linnean Society*, **97**: 709–717.

Miller RE, Rausher MD & Manos PS. (1999). Phylogenetic systematics of *Ipomoea* (*Convolvulaceae*) based on ITS and waxy sequences. *Systematic Botany*, 209–227.

Narbona E, Wang H, Ortiz PL, Arista M & Imbert E. (2018). Flower colour polymorphism in the Mediterranean Basin: Occurrence, maintenance and implications for speciation. *Plant Biology*, **20**: 8–20.

Nordborg M. (2001). Coalescent theory. In Balding DJ, Bishop M & Cannings C. (eds.). Handbook of Statistical Genetics. Pp. 179–212. Wiley.

Pastor J. (Ed.) (1992). Atlas Cromosómico de la Flora Vascular de Andalucia Occidental. Pp 283–284. Universidad de Sevilla.

Pau C. (1915). Sobre *Anagallis monelli* L. Boletín de la Sociedad Aragonesa de Ciencias Naturales, 14(5): 100–107.

Petit C, Bretagnolle F & Felber F. (1999). Evolutionary consequences of diploid–polyploid hybrid zones in wild species. *Trends in Ecology & Evolution*, **14(8)**: 306–311.

Pujadas A. (1997). *Anagallis* L. In: Castroviejo S, Aedo C, Laínz M, Morales R, Muñoz Garmendia F, Nieto Feliner G, Paiva J. (eds.) Flora iberica, V. Ebenaceae-Saxifragaceae. Pp. 57–62. Real Jardín Botánico, CSIC. Madrid.

Rambaut A, Suchard MA, Xie D & Drummond AJ (2014). Tracer v1.6 Available from http://beast.bio.ed.ac.uk/Tracer

Sampaio G. (1900). Estudos sobre a Flora dos Arredores do Porto. I. Primulaceae (Vent.) Annaes de Sciences Naturaes (Porto) 6: 51–62.

Schousboe P. (1800). Iagttagelser over Vextriget i Marokko. . Kjöbenhavn: Trykt for Fofatteren has K. H. Seidelin. Copenhague.

Semerikova SA & Semerikov VL. (2016). Phylogeny of firs (genus *Abies*, Pinaceae) based on multilocus nuclear markers (AFLP). *Russian Journal of Genetics*, **52(11)**: 1164–1175.

Sennen F. (1936). Plantes d' Espagne. 3ª parte. *Boletin de la Sociedad Ibérica de Ciencias Nat*urales, **35**: 17–32.

Shaw J, Lickey EB, Schilling EE & Small RL. (2007). Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare III. *American Journal of Botany*, **94**: 275–288.

Souissi A, Gagnaire PA, Bonhomme F & Bahri-Sfar L. (2017). Introgressive hybridization and morphological transgression in the contact zone between two Mediterranean Solea species. *Ecology and Evolution*, **7(5)**: 1394–1402.

Stamatakis A. (2014). RAxML Version 8: A tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies. *Bioinformatics*, **30**: 1312–1313.

Strauss SY & Whittall JB. (2006). Non-pollinator agents of selection on floral traits. In Harder LD & Barrett SCH (eds.). Ecology and Evolution of Flowers. Pp. 120–138. Oxford University Press, Oxford, UK.

Šveřepová G. (1972). Zur Zytotaxonomie der Gattung *Anagallis* L. (K cytotaxonomii rodu *Anagallis* L.). *Preslia*, Praha, **44(3)**, 219–226.

Šveřepová G. (1978). Artkreuzng von *Anagallis arvensis* und *Anagallis foemina* und Genese der Sektiom *Anagallis*. Folia Geobotanica et Phytotaxonomica, Praha, 13: 125-224.

Swofford DL. (2003). PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Symonds VV & Lloyd AM. (2003). An analysis of microsatellite loci in Arabidopsis thaliana: mutational dynamics and application. *Genetics*, **165**: 1475–1488.

Takahashi Y, Takakura K I & Kawata M. (2016). Spatial Distribution of Flower Color Induced by Interspecific Sexual Interaction. *PloS one*, **11(10)**: e0164381.

Talavera S, García Pérez L, Arista M & Ortiz PL. (1997). Números cromosomáticos de plantas occidentales 734-737. *Anales del Jardín Botánico de Madrid*, **55**: 136.

Talavera S, Gibbs PE, Fernández-Piedra MP & Ortiz-Herrera MA. (2001). Genetic control of self-incompatibility in *Anagallis monelli* (Primulaceae: Myrsinaceae). *Heredity,* **87**(5): 589.

Talavera M, Balao F, Casimiro-Soriguer R, Ortiz MÁ, Terrab A, Arista M, Ortiz PL, Stuessy T & Talavera S. (2011). Molecular phylogeny and systematics of the highly polymorphic *Rumex bucephalophorus* complex (Polygonaceae). *Molecular phylogenetics and evolution*, **61(3)**: 659–670.

Tamura K, Stecher G, Peterson D, Filipski A & Kumar S. (2013). MEGA 6: Molecular Evolutionary Genetics Analysis v. 6.0. *Molecular Biology and Evolution*, **30**: 2725–2729.

Taylor P. (1955). The genus *Anagallis* in tropical and South Africa. *Kew Bulletin*, **3**: 321–350.

Thompson JD, Gibson T & Higgins DG. (2002). Multiple sequence alignment using ClustalW and ClustalX. *Current protocols in bioinformatics,* 2 Unit 2.3.

Tournefort JP. (1719). Institutiones rei herbariae, ed.3, 1. Paris.

Valdés B. (1970). Números cromosómicos de algunas plantas españolas. *Boletin de la Real Sociedad Española de Historia Natural (Biología)*, **68**:193–197.

Viruel J, Forest F, Paun O, Chase MW, Devey D, Couto RS, Segarra-Moragues JG, Catalán P & Wilkin P. (2018). A nuclear Xdh phylogenetic analysis of yams (Dioscorea: Dioscoreaceae) congruent with plastid trees reveals a new Neotropical lineage. *Botanical Journal of the Linnean Society*, **187(2)**: 232–246.

Viruel J, Segarra-Moragues JG, Raz L, Forest F, Wilkin P, Sanmartín I & Catalán P. (2016). Late Cretaceous–Early Eocene origin of yams (*Dioscorea*, *Dioscoreaceae*) in the Laurasian Palaearctic and their subsequent Oligocene–Miocene diversification. *Journal of Biogeography*, **43(4)**: 750–762.

Volkova PA, Schanzer IA & Meschersky IV. (2013). Colour polymorphism in common primrose (*Primula vulgaris* Huds.): many colours–many species?. *Plant systematics and evolution*, **299(6)**: 1075–1087.

Wakeley J. (2009). *Coalescent theory: an introduction*. Roberts and Company Publishers, Greenwood Village, Colorado.

Wang H, Conchou L, Bessière JM, Cazals G, Schatz B & Imbert E. (2013). Flower color polymorphism in Iris lutescens (Iridaceae): biochemical analyses in light of plant–insect interactions. *Phytochemistry*, **94**: 123–134.

Wang H, Talavera M, Min Y, Flaven E & Imbert E. (2016). Neutral processes contribute to patterns of spatial variation for flower colour in the Mediterranean *Iris lutescens* (Iridaceae). *Annals of botany*, **117(6)**: 995–1007.

Wendel JF & Doyle JJ. (1998) Phylogenetic incongruence: window into genome history and molecular evolution. In: Soltis DE, Soltis PS & Doyle JJ. (eds). Molecular Systematics of Plants II. Pp 265-296. Springer, Boston, MA

White TJ, Bruns T, Lee S & Taylor J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications*, 18: 315–322.

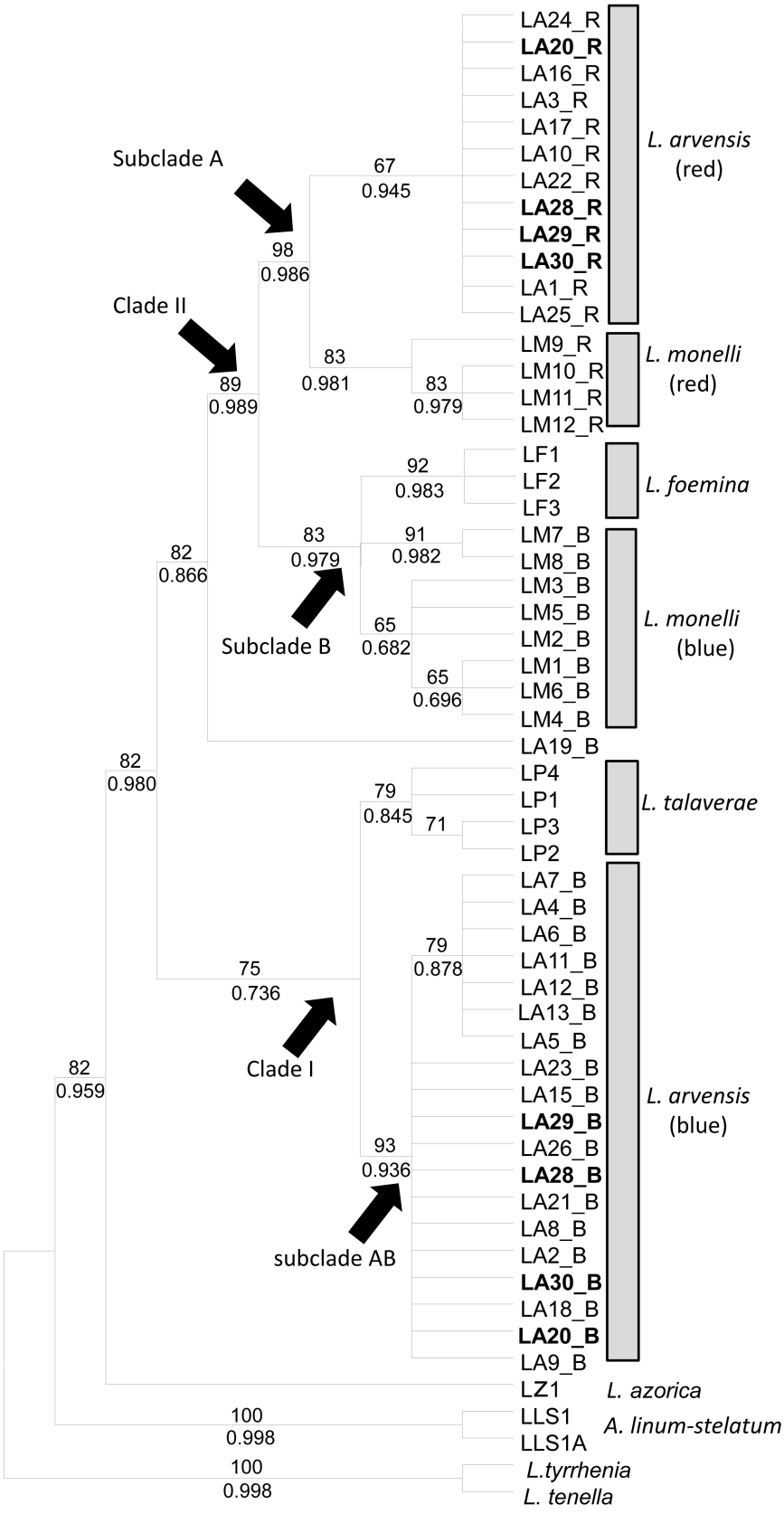
Willkomm HM. (1870). Primulaceae. In: Willkomm HM & Lamge JM (eds.). Prodromus Florae Hispanicae, 2. Pp. 635–650. Stuttgart

Wolf PG, Murray RA & Sipes SD. (1997). Species-independent, geographical structure of chloroplast DNA haplotypes in a montane herb *Ipomopsis* (Polemoniaceae). *Molecular Ecology*, **6**: 283–291.

Wolfe KH, Li WH & Sharp PM. (1987). Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. *Proceedings of the National Academy of Sciences*, **84(24)**: 9054–9058.

Yesson C, Toomey NH & Culham A. (2009). *Cyclamen*: time, sea and speciation biogeography using a temporally calibrated phylogeny. *Journal of Biogeography*, **36(7)**: 1234–1252.

Zhang CY, Wang FY, Yan HF, Hao G, Hu CM & Ge XJ. (2012). Testing DNA barcoding in closely related groups of *Lysimachia* L. (*Myrsinaceae*). *Molecular Ecology Resources*, **12**: 98–108.



Appendix 1. ML trees based on 32 ITS sequences of *L. arvensis* s.l. (including LA19_B), 12 sequences of *L. monelli* and 12 sequences of related taxa (see Table 1). Bootstrap values (> 50%) and posterior probabilities of Bayesian tree (> 0.6pp) are given above and below the branches respectively. The samples of *L. arvensis* from mixed populations highlighted in bold, when appear in different clades.

9. Discusión general.

Jiménez-López F.J.

DISCUSIÓN

El concepto de especie en plantas ha sido fuente de controversia para los biólogos evolutivos y un claro impedimento en los estudios botánicos de especiación. Las dificultades en la clasificación de especies en categorías discretas vienen suscitadas por la frecuente aparición de híbridos interespecíficos (Arnold 1992) y la amplia plasticidad fenotípica en algunos grupos de plantas (Ehrlich & Raven 1969; De Witt et al. 1998; Agrawal 2001; Lande 2015). Además, las múltiples evidencias de flujo génico encontradas entre diferentes especies de plantas, incrementan la dificultad en la categorización de las especies (Ellstrand 1992; Abbott 1992; Duminil & Di Michele 2009; Briggs & Walters 2016). Múltiples estudios indican que grupos discretos de individuos morfológicamente similares ocurren dentro de linajes de la misma especie sufriendo un significativo aislamiento reproductivo (Rieseberg et al. 2006). Sin embargo, estudios de genética de poblaciones indican que las tasas de migración dentro de las especies vegetales son más altas de las estimadas por valoraciones directas y que, incluso, en especies con bajo flujo génico, las poblaciones pueden evolucionar unidas a través de la propagación de alelos ventajosos (Whitlock 2003; McDaniel & Shaw 2005). Si bien muchas especies de plantas se mantienen unidas por el flujo génico y se mantienen separadas de otras especies por barreras reproductivas, existen excepciones. Las especies autógamas a menudo mantienen una cohesión genética y fenotípica (Rieseberg et al. 2006) porque tienen un mayor flujo genético dentro de las especies de lo que se había creído (Bakker et al. 2006), y un intercambio polínico restringido entre linajes impide la hibridación interespecífica. Sin embargo, el aislamiento reproductivo entre especies puede ser incompleto, particularmente en grupos que han sufrido recientemente eventos de especiación múltiple, lo que dificulta su identificación como especies (Levin 1985; Van der Niet et al. 2006).

Uno de los caracteres florales más conspicuos es el color floral que puede promover la diferenciación de grupos de plantas o poblaciones dentro de una especie, por limitar el flujo génico entre ellas (Servedio et al. 2011). El color de las flores se debe fundamentalmente a la presencia de pigmentos, siendo las antocianinas los más comunes y responsables de los colores rojos, anaranjados, azules y violáceos (Grotewold 2006). La mayoría de las variaciones del color de las flores se deben a cambios en las antocianinas y las transiciones más frecuentes van del azul-púrpura al rojo-naranja, por la producción de antocianinas menos hidrogenadas, o de flores pigmentadas a blancas por la pérdida de antocianinas (ver Rausher 2008). El color actúa como reclamo selectivo de visitantes (Stebbins 1974; Melendez-Ackerman & Campbell 1998; Weiss 2001), los cuales pueden asociarlo con la calidad de la recompensa floral (Waser & Price 1985; Melendez-Ackerman et al. 1997) y mostrar

preferencias sobre uno de ellos. Los cambios en el color floral pueden promover la especiación por variaciones en el espectro de polinizadores que reducen el flujo génico entre las distintas formas de color (Hodges et al. 2002; Bradshaw & Schemske 2003; pero ver Cooley et al. 2008 y Smith et al. 2008). Aunque algunos estudios muestran que ciertos polinizadores no discriminan entre colores (Mogford 1978; Miller 1981), muchos otros ponen de manifiesto la existencia de selección direccional (Levin & Brack 1995; Campbell et al. 1997; Morgan & Schoen 1997, Waser & Price 1981; Jones & Reithel 2001). De hecho el color de las flores es uno de los componentes de los conocidos "síndromes de polinización" (Stebbins 1974; Faegri & Van der Pijl 1979). Como ejemplo encontramos el significado de las flores rojas en el contexto de la polinización ornitófila; tal color sería invisible a las abejas y atractivo para los colibríes (Raven 1972). Hasta la fecha solo existe un caso bien conocido en el que un cambio en el color floral parece haber desencadenado un proceso de especiación. Es el ejemplo de Mimulus lewisi y M. cardinalis (Ramsey et al. 2003). El cambio de pétalos rosas (*Mimulus lewisii)* a rojos (*M. cardinalis)* indujo un cambio en la frecuencia de visitas de los distintos tipos de polinizadores lo que originó que los cruzamientos se llevasen a cabo preferentemente dentro de un mismo color, desencadenando el proceso de especiación (Ramsey et al. 2003).

Sin embargo, el color floral también puede originar especiación debido a que puede producir diferencias en el fitness asociadas a otros caracteres correlacionados (eg. Levin & Brack 1995; Armbruster et al. 1997; Armbruster 2002; Frey 2004). Muchos enzimas necesarios para la síntesis de antocianinas (principales responsables del color) también intervienen en la de otros flavonoides que protegen de radiaciones UV, patógenos y herbívoros, y confieren mayor tolerancia al estrés ambiental (Koes et al. 1994, 2005; Johnson et al. 2008). Una revisión de los efectos pleiotrópicos sobre el color de las flores señala que diferencias en este carácter suelen estar asociadas a diferencias en la supervivencia de las plántulas, la producción de flores y de semillas, la biomasa vegetativa y la resistencia a herbívoros y patógenos (Strauss & Whittall 2006). Por tanto, plantas con distintos tipos de antocianinas pueden tener una tolerancia diferencial a los ambientes (ejp. Schemske & Bierzychudek 2001), lo que influiría en su patrón de distribución y finalmente conduciría a especiación si el flujo génico entre esos ambientes es pequeño (Servedio et al. 2011).

Por último, diferencias en el color floral pueden surgir posteriormente como mecanismo que refuerce una especiación incipiente para disminuir la posibilidad de cruzamiento entre esas especies hermanas. (Pfennig & Pfennig 2010; McEwen & Vamosi 2010; Hopkins & Rausher 2012; Hopkins 2013; Grossenbacher & Stanton 2014). Este proceso se denomina "Reinforcement" y se ha descrito hasta la fecha

únicamente en *Phlox* (Hopkins & Rausher 2011; 2012) y en *Petunia* (Quattrocchio et al. 1999).

La posibilidad de que en *Lysimachia arvensis*, especie con dos morfotipos de color, el color floral estuviese iniciando un proceso de especiación se ha evaluado en esta tesis doctoral. La información con la que contábamos indicaba que los morfos tenían un patrón geográfico de distribución, con el azul mejor representado y con un mayor fitness en los ambientes secos Mediterráneos, en los que muchas poblaciones son mixtas (Arista et al. 2013). También se había descrito la polinización por abejas solitarias de pequeño tamaño que diferenciaban visualmente los dos colores florales y que mostraban una clara preferencia por el morfo azul en las poblaciones mixtas (Ortiz et al. 2015). Estos datos sugerían la posibilidad de que los cruzamientos entre morfos de color no se estaban produciendo con la misma frecuencia que dentro de los morfos, un factor clave para el desencadenamiento de un proceso de especiación. A partir de esta información, hemos estudiado diferentes aspectos relacionados con esta posibilidad: cómo se segrega el color floral, cómo se están cruzando las plantas en las poblaciones naturales, qué tipos de barreras de cruzamiento existen entre los morfos de color, y cual es su diversidad genética y su posición filogenética dentro del conjunto de especies con las que están emparentados. Tal y como se discute a continuación, todos los resultados obtenidos sugieren que los dos morfos de color son en realidad especies bien definidas en las que el color no parece haber sido el desencadenante de su separación aunque sí está teniendo un papel importante como mecanismo de refuerzo junto a otros caracteres menos conspicuos.

Hasta la fecha, se tenía una información muy difusa y en gran medida errónea sobre cómo se segregaba el color floral en *L. arvensis*, ya que se pensaba que el alelo para el color rojo era dominante sobre el azul (Marsden-Jones & Weiss 1938). Esta suposición explicaba claramente el porqué en las poblaciones mixtas de esta especie no solían aparecer otros colores florales diferentes. Las escasas ocasiones en las que se habían encontrados plantas con flores de otro color, se habían interpretado de maneras diversas. De hecho, mutaciones ocasionales en la ruta biosintética de las antocianinas son comunes en muchas especies de angiospermas y originan fenotipos de color que suelen desaparecer en una o dos generaciones. En *L. arvensis* aunque la alteración en la ruta biosintética de los pigmentos responsables de color floral no se ha estudiado directamente en esta tesis doctoral (en la actualidad está siendo abordada por el grupo de investigación de esta tesis), los cruces realizados a mano entre ambos linajes de color, y entre la F1 obtenida de esos cruces consigo misma, claramente indican que la información de partida era errónea (Capítulo 2). De hecho, el cruce entre linajes puros de color siempre origina una descendencia homogénea de color intermedio, salmón, que raramente aparece en las poblaciones que hemos

muestreado. Este resultado unido al intenso muestreo de poblaciones que se ha realizado en esta tesis tiene una gran importancia, ya que claramente nos indica la baja tasa de cruzamiento entre estos dos linajes en la naturaleza.

Nuestros resultados también muestran que los dos linajes presentan diferencias sutiles pero consistentes en distintos aspectos ecológicos y morfológicos. Así nuestros datos apoyan claramente una mejor adaptación del linaje azul al clima mediterráneo ya que las semillas germinan antes, sobreviven más plántulas y las plantas florecen antes que en el linaje rojo (Capítulo 4; ver resultados previos de Arista et al. 2013). En los ambientes Mediterráneos es frecuente que la precipitación otoñal ocurra en pulsos (Chesson et al. 2004), por lo que en las especies anuales se produce una germinación escalonada que favorece a aquellos individuos que han germinado antes al permitir el desarrollo de raíces profundas durante el periodo de escasez de agua (Schenk & Jackson 2002). Además, el momento de la germinación es el primer paso importante en el desarrollo de una planta e influye en todos los rasgos del ciclo vital (Manzano-Piedras et al. 2014). En las plantas anuales, una germinación temprana cuando el agua está disponible permite además un período de floración prolongado y, en climas estacionales como el Mediterráneo, esto es ventajoso ya que asegura la reproducción de las plantas (Rodríguez-Pérez & Traveset 2016). Sin embargo, no es solo que las semillas del linaje azul germinen antes, sino que las plántulas también tuvieron un desarrollo más rápido alcanzando la madurez reproductiva mucho antes que las del linaje rojo, lo que origina un cierto desfase en la fenología floral (Capítulos 4 y 7). Este desfase entre linajes disminuye la posibilidad de cruzamiento entre ellos y limita el flujo de polen en poblaciones simpátricas, actuando como una importante barrera precigótica al flujo génico (Martin & Willis 2007; Botes et al. 2008, Brys et al. 2013).

En las poblaciones mixtas Mediterráneas se ha observado cierta segregación espacial que puede aliviar el efecto negativo del clima Mediterráneo para el linaje rojo, situándose las escasas plantas de flores rojas en lugares más umbríos y húmedos (Arista et al. 2013). Cabría esperar que, esta mala adaptación del linaje rojo al Mediterráneo junto con las escasas visitas que recibe de los polinizadores llevaran a su desaparición en este área. Sin embargo, durante esta tesis doctoral hemos tenido oportunidad de visitar repetidamente diversas poblaciones mixtas y siempre hemos encontrado algunas plantas con flores rojas. Una posibilidad para que el linaje rojo se mantuviera en las poblaciones naturales a pesar de estar seleccionado en contra tanto abiótica como bióticamente es la producción de semillas por autopolinización. Para ello sería necesario que las plantas fuesen capaces de producir semillas por autopolinización automática y no se vieran muy afectadas por depresión por endogamia (Jain 1976; Schemske & Lande 1985; Charlesworth & Charlesworth 1987; Holsinger 1988). La hercogamia, o separación espacial de anteras y estigma, que se

había observado en las flores de *L. arvensis*, podría suponer una dificultad a la autogamia tal y como ocurre en muchas otras especies (Webb & Lloyd 1986).

El estudio profundo de la hercogamia en los linajes de *L. arvensis* mostró la existencia de dos tipos secuenciales de hercogamia, aunque con patrones bien diferentes. El linaje azul fue mucho más variable en este carácter, con poblaciones cuyas plantas tienen una hercogamia marcada que desfavorece completamente la autogamia y otras, cuya hercogamia lateral favorece la autogamia ya desde el primer día de apertura floral. Sin embargo, el linaje rojo fue mucho más homogéneo a este respecto, con una hercogamia lateral fuerte el primer día y reversa el segundo día (Capítulo 6). Esta combinación de hercogamia implica que el linaje rojo es totalmente dependiente de la actividad de los polinizadores durante el primer día de la antesis, pero asegura la reproducción en caso de que éstos fallen ("delayed selfing" Lloyd & Shoen 1992). Estos patrones de hercogamia se suman una vez más a la pléyade de pequeñas diferencias entre linajes que sugieren aislamiento entre ellos. Dado que la hercogamia es un carácter que muestra variación, es heredable (Capítulo 5) y está sujeto a selección por parte de los polinizadores, cabría esperar que un defecto continuo de polinizadores en el linaje rojo en el Mediterráneo hubiese originado la pérdida completa de la hercogamia favoreciendo la autopolinización automática desde el primer día de apertura floral. Dado que esto no ocurre, cabe pensar que existe alguna desventaja para la pérdida completa de la hercogamia en el linaje rojo. Es destacable que los niveles de depresión por endogamia en el Mediterráneo fueron muy diferentes para los dos linajes (Capítulo 4). Los valores encontrados en el linaje azul se ajustaron a los de las especies con sistemas mixtos de reproducción, lo que concuerda con la variabilidad encontrada en la hercogamia. Los valores de depresión por endogamia en el linaje rojo fueron poco variables, relativamente altos, y propios de las especies xenógamas. Se podría pensar que la alta depresión por endogamia de este linaje dificulta la pérdida completa de hercogamia, por lo que las plantas apostarían primero por producir semillas xenógamas, dejando la producción de semillas autógamas como último recurso de aseguramiento reproductivo.

Por tanto, aunque la hercogamia que muestra el linaje rojo no impide la autodeposición de polen, los valores altos de depresión por endogamia podrían indicar que el linaje rojo no se reproduce por autogamia. Sin embargo, los estudios moleculares mostraron que sus poblaciones tienen una diversidad genética más baja, menor heterocigosidad observada (Ho) y menor número de alelos que el linaje azul, especialmente en la región Mediterránea (Capítulo 4). Dado que los sistemas de cruzamiento repercuten en la diversidad genética, se espera que los sistemas autógamos muestren una diversidad mucho menor que los xenógamos (Charlesworth & Wright 2001). Según nuestros resultados, las diferencias en la diversidad genética

entre ambos linajes apoyan una estrategia de autopolinización en las plantas de flores rojas en el Mediterráneo a pesar de la disminución del 60% de fitness de la progenie autógama por depresión de endogamia. Todo implica que alguna descendencia autógama se está reclutando en las poblaciones, posiblemente en baja cantidad. El aseguramiento reproductivo podría por tanto estar manteniendo al linaje rojo en las poblaciones Mediterráneas, disminuyendo el flujo génico con otras plantas en general y con el linaje azul en particular.

Es interesante destacar que a pesar de todos estos indicios de separación entre linajes, los polinizadores realizan visitas secuenciales entre ellos (Capítulo 7) por lo que cabría esperar cierto flujo polínico. Además, los cruces manuales entre ellos realizados en esta tesis dieron lugar a una descendencia F1 viable que incluso mostró mayor vigor que los parentales, aunque este vigor desapareció en la F2. Estas observaciones chocan con la ausencia de híbridos de primera generación en la naturaleza por lo que era necesario el estudio de las múltiples barreras precigóticas y postcigóticas que podían operar en este sistema. Hay que destacar que este es uno de los primeros estudios que afronta una cuantificación exhaustiva de las barreras de aislamiento entre linajes ya que ha incluido tanto las barreras ecogeográficas como cada una de las pequeñas barreras ecológicas y genéticas que actúan en la naturaleza. Hemos encontrado que el aislamiento casi completo entre linajes se debe al concurso de múltiples barreras precigóticas que actúan secuencialmente (Capítulo 7), y que hacen que el flujo polínico entre ellos pueda ser cada vez más pequeño a lo largo del ciclo reproductivo de la planta. De hecho, las barreras postcigóticas fueron incluso negativas, algo que ha sido descrito previamente (Lowry et al. 2008; Baack et al. 2015). La intensidad de las barreras precigóticas hace que prácticamente no se produzcan híbridos en la naturaleza, por lo que existe una menor presión para el desarrollo de las barreras postcigóticas. Las diferencias genéticas entre linajes hacen que la F1 muestre un vigor híbrido que es posiblemente consecuencia de su mayor heterocigosidad, pero que resulta en una disminución del fitness en la F2, por lo que cabría esperar la aparición de mecanismos de refuerzo en las poblaciones donde los linajes coexisten.

Todos los resultados que hemos ido obteniendo han indicado que en menor o mayor medida los dos linajes de color están aislados. Cuando estudiamos la filogenia del grupo de especies en la que se incluye *L. arvensis*, encontramos sorprendentemente que los dos linajes de color se encuentran en clados diferentes (Capítulo 8). Un clado incluye al linaje azul junto a *L. talaverae*, una especie también de flores azules, y otro que contiene dos subclados, uno de flores rojas que incluye al linaje rojo y a *L. collina*, y otro de flores azules que incluye a *L. monelli* y a *L. foemina* (Capítulo 8). Aunque no tenemos certeza de cual era el color floral del ancestro de los dos clados en los que se

encuadran los linajes de *L. arvensis*, por parsimonia cabría esperar un ancestro de flores azules. De hecho, es conocido que la transición desde el color azul al rojo es bastante frecuente debido a la inactivación de una rama de la ruta de las antocianinas (Rausher 2008) y en los linajes estudiados de otros grupos vegetales, las especies de flores rojas derivan habitualmente de las de flores azules (kay et al. 2005; Wilson et al. 2007; Rausher 2008; Streisfeld & Rausher 2009; Wessinger & Rausher 2012). En nuestro estudio ese ancestro de flores azules originaría dos clados, uno completamente azul (que incluye al linaje azul de *L. arvensis*) y otro que posteriomente se separó en un subclado azul y otro rojo (que incluye al linaje rojo de *L. arvensis*). Este resultado nos indica que los dos linajes de *L. arvensis* son en realidad especies bien diferenciadas, que hemos nombrado aquí como *L. arvensis* (linaje rojo) *y L. loeflingii* (linaje azul), pero además, también nos indica que el color floral no puede haber sido el desencadenante de la especiación.

De hecho, la disposición de cada linaje en la reconstrucción filogenética los relaciona más próximos evolutivamente a otras especies diploides, lo que sugiere que tienen orígenes independientes. Buena parte de los eventos de especiación en las plantas implican procesos de hibridación y posterior modificación de genomas divergentes (Stebbins 1971). En ese sentido se han definido dos tipos de especiación híbrida: homoploide y poliploide. La homoploide se refiere al origen de un nuevo linaje híbrido sin cambio en el número de cromosomas, mientras que la poliploide implica la duplicación completa del genoma tras la hibridación (alopoliploidía). Además, se pueden producir nuevas especies no por hibridación, sino por duplicación del genoma lo que también origina poliploidía (autopoliploidía). La configuración observada en la reconstrucción filogenética hace pensar que ambos linajes de *L. arvensis*, que son tetraploides, se han podido originar por autopoliploidía. *Lysimachia arvensis* (linaje rojo) podría derivar de *L. collina* (diploide con flores rojas) y *L. loeflingii* podría derivar de *L. talaverae* (diploide con flores azules). Esto indicaría la presencia de múltiples eventos de poliploidización en el clado estudiado, algo que ya se ha encontrado previamente en otros géneros (ejp. Rieseberg et al. 2003; Wang et al. 2006; Wood et al. 2009; Soltis et al. 2015; Padilla-García et al. 2018). Sin embargo, este aspecto necesitaría de un estudio más dirigido.

Por tanto, en el Mediterráneo nos encontramos con poblaciones donde conviven estas dos especies, relativamente recientes y muy cercanas filogenéticamente (Capítulo 8) y que comparten polinizadores. La suma de barreras origina un 80% de aislamiento reproductivo entre ellas. En este aislamiento, el color floral parece jugar un papel importante por estar implicado directamente en el desarrollo de al menos dos de las barreras estudiadas. La diferencia de color influye en la antesis diaria de las flores que, como en otros casos estudiados (He et al. 2005; Mu et al. 2010; ver revisión en

van Doorn & van Meeteren 2003), parece guardar relación con la temperatura. Las flores azules se calientan antes y tardan más en enfriarse por lo que su periodo diario de apertura es mas largo que el de las rojas. De esta manera hay ciertos momentos del día donde solo hay flores azules abiertas lo que impide el flujo génico entre especies. Pero lo más importante es que las abejas tienen una marcada preferencia por las flores azules, y se mueven con mucha mayor preferencia entre las flores azules que entre las azules y rojas o entre las rojas. Estos movimientos no aleatorios contribuyen de manera muy importante al aislamiento entre estas dos especies. La posible asociación entre el color floral y la tolerancia a ambientes secos como consecuencia de un efecto pleiotrópico relacionado con la presencia de flavonoides ha sido también sugerido (Arista et al. 2013), pero este aspecto requiere mayor atención. Todo ello sugiere que el color floral puede estar funcionando como un mecanismo de refuerzo que favorece la evolución de barreras precigóticas intensas entre estas especies emergentes (Hopkins & Rausher 2012; Hopkins 2013).

FUTURAS DIRECCIONES

Esta tesis doctoral deja abiertos muchos interrogantes relacionados con las especies estudiadas o bien con otras especies hermanas que merecerían estudios más profundos. Algunos de estos interrogantes ya han sido señalados a lo largo de esta discusión e incluso ya se han comenzado a abordar. Sería interesante conocer los genes responsables de la diferencia de color entre *L. arvensis* y L. *loeflingii* mediante el estudio de la expresión génica a través del transcriptoma de la ruta de las antocianinas. Este estudio nos permitiría además cuantificar otros compuestos como las flavonas y flavonoles que se originan en esa misma ruta biosintética y que tienen relación directa con la protección frente al estrés ambiental o la herbivoría, lo que nos explicaría el patrón de distribución de estas especies. Otro aspecto que creemos de interés es el estudio filogeográfico de estas dos especies, que combinado con modelos de distribución de nicho nos ayudaría a entender el origen geográfico de las mismas y nos permitiría pronosticar su distribución futura. Esta información podría ser de gran interés en un futuro entorno de cambio climático, donde el aumento de la aridez podría conllevar un aumento del área de distribución de *L. loeflingii* y una disminución del área de *L. arvensis*. Otro aspecto de interés, es el papel de la poliploidía en el origen de las especies del clado de *L. arvensis*. Para las especies relacionadas con *L. arvensis* aquí estudiadas se conoce el número de cromosomas, pero no se ha estudiado en profundidad el cariotipo de ninguna de ellas que podría ser abordado mediante técnicas de FISH. La posibilidad de flujo génico entre *L. arvensis*, *L. loeflingii* y *L. foemina* es otro aspecto que merecería la pena abordar ya que las tres

especies aparecen conviviendo en muchas poblaciones, e incluso *L. foemina* estuvo incluida como subespecie de *L. arvensis* durante mucho tiempo, hasta que los análisis moleculares evidenciaran su posición más cercana a *L. monelli*. Por último, y en base a los resultados obtenidos en la filogenia, se abre el interrogante sobre el papel que ha jugado el color en la separación de *L. monelli* y *L. collina*, dos especies hermanas que hasta ahora han estado bajo la misma especie y que sabemos por estudios preliminares que son completamente interfértiles.

REFERENCIAS

Abbott RJ. (1992). Plant invasions, interspecific hybridization and the evolution of new plant taxa. *Trends in ecology & evolution*, **7**: 401-405.

Agrawal A.A. (2001). Phenotypic plasticity in the interactions and evolution of species. Science, **294**: 321-326.

Arista M, Talavera M, Berjano R. & Ortiz PL. (2013). Abiotic factors may explain the geographical distribution of flower colour morphs and the maintenance of colour polymorphism in the scarlet pimpernel. *Journal of Ecology*, **101**: 1613-1622.

Armbruster P, Bradshaw WE. & Holzapfel CM. (1997). Evolution of the genetic architecture underlying fitness in the pitcher-plant mosquito, *Wyeomyia smithii*. *Evolution*, **51**: 451-458.

Armbruster WS. (2002). Can indirect selection and genetic context contribute to trait diversification? A transition-probability study of blossom-colour evolution in two genera. *Journal of Evolutionary Biology*, **15**: 468-486.

Arnold ML. (1992). Natural hybridization as an evolutionary process. *Annual review of Ecology and Systematics*, **23**: 237-261.

Baack E, Melo MC, Rieseberg LH. & Ortiz-Barrientos D. (2015). The origins of reproductive isolation in plants. *New Phytologist*, **207**: 968-984.

Bakker EG, Stahl EA, Toomajian C, Nordborg M, Kreitman M. & Bergelson J. (2006). Distribution of genetic variation within and among local populations of *Arabidopsis thaliana* over its species range. *Molecular Ecology*, **15**: 1405-1418.

Botes C, Johnson SD. & Cowling RM. (2008). Coexistence of succulent tree aloes: partitioning of bird pollinators by floral traits and flowering phenology. *Oikos*, **117**: 875–882.

Bradshaw HD. & Schemske DW. (2003). Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. *Nature*, **426**: 176.

Briggs D. & Walters SM. (2016). Plant variation and evolution. Cambridge University Press.

Brys R, Broeck AV, Mergeay J. & Jacquemyn H. (2013). The contribution of mating system variation to reproductive isolation in two closely related *Centaurium* species (Gentianaceae) with a generalized flower morphology. *Evolution*, **68**: 1281–1293.

Campbell DR, Waser NM. & Melendez-Ackerman E.J. (1997). Analyzing pollinator-mediated selection in a plant hybrid zone: hummingbird visitation patterns on three spatial scales. *The American Naturalist*, **149**: 295-315.

Charlesworth D. & Charlesworth B. (1987). Inbreeding depression and its evolutionary consequences. *Annual review of ecology and systematics*, **18**: 237-268.

Charlesworth D. & Wright S.I. (2001). Breeding systems and genome evolution. *Current Opinion Genetic Development*, **11**: 685–690.

Chesson P, Gebauer RLE, Schwinning S, Huntly N, Wiegand K, Ernest MSK, Sher A, Novoplansky A. & Weltzin JF. (2004). Resource pulses, species interactions, and diversity maintenance in arid and semi-arid environments. *Oecologia*, **141**: 236-253.

Cooley AM, Carvallo G. & Willis JH. (2008). Is floral diversification associated with pollinator divergence? Flower shape, flower colour and pollinator preference in Chilean *Mimulus*. *Annals of Botany*, **101**: 641-650.

DeWitt TJ, Sih A. & Wilson DS. (1998). Costs and limits of phenotypic plasticity. *Trends in ecology & evolution*, **13**: 77-81.

Duminil J. & Di Michele M. (2009). Plant species delimitation: a comparison of morphological and molecular markers. *Plant Biosystems*, **143**: 528-542.

Ehrlich P.R. & Raven P.H. (1969). Differentiation of populations. Science, 1228-1232.

Ellstrand N.C. (1992). Gene flow by pollen: implications for plant conservation genetics. Oikos, 77-86.

Faegri K. & Van der Pijl. (1979). The principles of pollination ecology. Third revised edition. Pergamon Press. Oxford. England

Frey FM. (2004). Opposing natural selection from herbivores and pathogens may maintain floral-color variation in *Claytonia virginica* (Portulacaceae). *Evolution*, **58**: 2426-2437.

Grossenbacher DL. & Stanton ML. (2014). Pollinator-mediated competition influences selection for flower-color displacement in sympatric monkeyflowers. *American journal of botany*, **101**: 1915-1924.

Grotewold E. (2006). The genetics and biochemistry of floral pigments. *Annual Revision of Plant Biology*, **57**: 761-780.

He YP, Duan YW, Liu JQ. & Smith WK. (2005). Floral closure in response to temperature and pollination in *Gentiana straminea* Maxim. (Gentianaceae), an alpine perennial in the Qinghai-Tibetan Plateau. *Plant Systematics and Evolution*, **256**: 17-33.

Hodges SA, Whittall JB, Fulton M. & Yang JY. (2002). Genetics of floral traits influencing reproductive isolation between *Aquilegia formosa* and *Aquilegia pubescens*. *The American naturalist*, **159**: S51-S60.

Holsinger KE. (1988). Inbreeding depression doesn't matter: the genetic basis of mating-system evolution. *Evolution*, **42**: 1235-1244.

Hopkins R. (2013). Reinforcement in plants. *New Phytologist*, **197(4)**, 1095-1103.

Hopkins R. & Rausher MD. (2011). Identification of two genes causing reinforcement in the Texas wildflower *Phlox drummondii*. *Nature*, **469**: 411.

Hopkins R. & Rausher MD. (2012). Pollinator-mediated selection on flower color allele drives reinforcement. *Science*, **335**: 1090-1092.

Jain SK. (1976). The evolution of inbreeding in plants. *Annual review of ecology & systematics*, **7**: 469–495.

Johnson ET, Berhow MA. & Dowd PF. (2008) Colored and white sectors from star-patterned *Petunia* flowers display differential resistance to corn earworm and cabbage looper larvae. *Journal of Chemical Ecology*, **34**: 757–765.

Jones KN. & Reithel J.S. (2001). Pollinator-mediated selection on a flower color polymorphism in experimental populations of *Antirrhinum* (Scrophulariaceae). *American Journal of Botany*, **88**: 447-454.

Kay KM, Reeves PA, Olmstead RG. & Schemske DW. (2005). Rapid speciation and the evolution of hummingbird pollination in neotropical *Costus* subgenus *costus* (Costaceae): evidence from nrDNA ITS and ETS sequences. *American Journal of Botany*, **92**: 1899–1910.

Koes RE, Quattrocchio F. & Mol JN. (1994). The flavonoid biosynthetic pathway in plants: function and evolution. *BioEssays*, **16**: 123-132.

Koes R, Verweij W. & Quattrocchio F. (2005). Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. *Trends in plant science*, **10**: 236-242.

Lande R. (2015). Evolution of phenotypic plasticity in colonizing species. *Molecular ecology*, **24**: 2038-2045.

Levin DA. (1985). Reproductive character displacement in *Phlox*. *Evolution*, **39**: 1275-1281.

Levin DA. & Brack ET. (1995). Natural selection against white petals in *Phlox*. *Evolution*, **49**: 1017-1022.

Lloyd DG. & Shoen DJ. (1992). Self- and cross-fertilization in plants. I. Functional dimensions. *International journal plant sciences*, **153**: 358-369.

Lowry DB, Modliszewski JL, Wright KM, Wu CA. & Willis JH. (2008). The strength and genetic basis of reproductive isolating barriers in flowering plants. *Philosophical Transactions of the Royal Society B*, **363**: 3009-3021

Manzano-Piedras E, Marcer A, Alonso-Blanco C. & Picó FX. (2014). Deciphering the adjustment between environment and life history in annuals: lessons from a geographically-explicit approach in *Arabidopsis thaliana*. *PloS one*, 9: e87836.

Marsden-Jones EM. & Weiss FE. (1938, June). The essential differences between *Anagallis arvensis* Linn. and *Anagallis foemina* MIll. In Proceedings of the Linnean Society of London. **150**: 146-155. Oxford, UK: Blackwell Publishing Ltd.

Martin NH. & Willis JH. (2007). Ecological divergence associated with mating system causes nearly complete reproductive isolation between sympatric *Mimulus* species. *Evolution*, **61**: 68–82.

McDaniel SF. & Shaw AJ. (2005). Selective sweeps and intercontinental migration in the cosmopolitan moss *Ceratodon purpureus* (Hedw.) Brid. *Molecular Ecology*, **14**: 1121-1132.

McEwen JR. & Vamosi JC. (2010). Floral colour versus phylogeny in structuring subalpine flowering communities. *Proceedings of the Royal Society of London B*, rspb20100501.

Meléndez-Ackerman E, Campbell DR. & Waser NM. (1997). Hummingbird behavior and mechanisms of selection on flower color in *Ipomopsis*. *Ecology*, **78**: 2532-2541.

Meléndez-Ackerman E. & Campbell DR. (1998). Adaptive significance of flower color and inter-trait correlations in an *Ipomopsis* hybrid zone. *Evolution*, **52**: 1293-1303.

Miller RB. (1981). Hawkmoths and the geographic patterns of floral variation in *Aquilegia caerulea*. *Evolution*, **35**: 763-774.

Mogford DJ. (1978). Pollination and flower colour polymorphism, with special reference to *Cirsium palustre*. The pollination of flowers by insects.

Morgan MT, Schoen DJ. & Bataillon TM. (1997). The evolution of self-fertilization in perennials. *The American Naturalist*, **150**: 618-638.

Mu J, Li G. & Sun S. (2010). Petal color, flower temperature, and behavior in an alpine annual herb, *Gentiana leucomelaena* (Gentianaceae). *Arctic, Antarctic, and Alpine Research*, **42**: 219-226.

Ortiz PL, Berjano R, Talavera M, Rodríguez-Zayas L. & Arista M. (2015). Flower colour polymorphism in *Lysimachia arvensis*: How is the red morph maintained in Mediterranean environments?. *Perspectives in Plant Ecology, Evolution and Systematics*, **17**: 142-150.

Padilla-García N, Rojas-Andrés BM, López-González N, Castro M, Castro S, Loureiro J, Albache DC, Machon N. & Martínez-Ortega MM. (2018). The challenge of species delimitation in the diploid-polyploid complex *Veronica* subsection Pentasepalae. *Molecular phylogenetics and evolution*, **119**: 196-209.

Pfennig, DW. & Pfennig KS. (2010). Character displacement and the origins of diversity. *The american naturalist*, **176**: S26-S44.

Quattrocchio F, Wing J, van der Woude K, Souer E, de Vetten N, Mol J. & Koes R. (1999). Molecular analysis of the anthocyanin gene of petunia and its role in the evolution of flower color. *The Plant Cell*, **11**: 1433-1444.

Ramsey J, Bradshaw HD. & Schemske DW. (2003). Components of reproductive isolation between the monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). *Evolution*, **57**: 1520-1534.

Rausher MD. (2008). Evolutionary transitions in floral color. *International Journal of Plant Sciences*, **169**: 7-21.

Raven PH. (1972). Why are bird-visited flowers predominantly red? *Evolution*, **26**: 674-674.

Rieseberg LH, Raymond O, Rosenthal DM, Lai Z, Livingstone K, Nakazato T, Durphy JL, Schwarzbach AE, Donovan LA. & Lexer C. (2003). Major ecological transitions in wild sunflowers facilitated by hybridization. *Science*, **301**: 1211-1216.

Rieseberg LH, Wood TE. & Baack EJ. (2006). The nature of plant species. *Nature*, **440**: 524.

Rodríguez-Pérez J. & Traveset A. (2016). Effects of flowering phenology and synchrony on the reproductive success of a long-flowering shrub. AoB Plants, 8: plw007.

Schemske DW. & Lande R. (1985). The evolution of self-fertilization and inbreeding depression in plants. II. Empirical observations. *Evolution*, **39**: 41-52.

Schemske DW. & Bierzychudek P. (2001). Perspective: evolution of flower color in the desert annual *Linanthus parryae*: Wright revisited. *Evolution*, **55**: 1269-1282.

Schenk JH. & Jackson RB. (2002). The global biogeography of roots. *Ecology monographs*, **72**: 311–328.

Servedio MR, Van Doorm GS, Kopp M, Frame AM. & Nosil P. (2011). Magic traits in speciation: 'magic' but not rare?. *Trends in ecology & evolution*, **26**: 389-397.

Smith SD, Ané C, & Baum DA. (2008). The role of pollinator shifts in the floral diversification of *Iochroma* (Solanaceae). *Evolution: International Journal of Organic Evolution*, **62**: 793-806.

Soltis PS, Marchant DB, Van de Peer Y. & Soltis DE. (2015). Polyploidy and genome evolution in plants. *Current opinion in genetics & development*, **35**: 119-125.

Stebbins GL. (1971). Adaptive radiation of reproductive characteristics in angiosperms, II: seeds and seedlings. *Annual Review of Ecology and Systematics*, **2**: 237-260.

Stebbins GL. (1974). Flowering plants: evolution above the species level. London: Arnold xviii, 399p. Illustrations. General (KR, 197500089).

Strauss SY. & Whittall JB. (2006). Non-pollinator agents of selection on floral traits. *Ecology and evolution of flowers*, 1439, 120.

Streisfeld MA. & Rausher MD. (2009). Genetic changes contributing to the parallel evolution of red floral pigmentation among *Ipomoea* species. *New Phytologist*, **183**: 751-763.

Van der Niet T, Johnson SD. & Linder HP. (2006). Macroevolutionary data suggest a role for reinforcement in pollination system shifts. *Evolution*, **60**: 1596-1601.

Van Doorn WG. & Van Meeteren U. (2003). Flower opening and closure: a review. *Journal of experimental botany*, **54**: 1801-1812.

Wang J, Tian L, Lee HS. & Chen ZJ. (2006). Non-additive regulation of FRI and FLC loci mediates flowering-time variation in *Arabidopsis* allopolyploids. *Genetics*, **173**: 965.

Waser NM. & Price MV. (1981). Pollinator choice and stabilizing selection for flower color in *Delphinium nelsonii*. *Evolution*, **35**: 376-390.

Waser NM. & Price MV. (1985). Reciprocal transplant experiments with *Delphinium nelsonii* (Ranunculaceae): evidence for local adaptation. *American Journal of Botany*, **72**: 1726-1732.

Webb CJ. & Lloyd DG. (1986). The avoidance of interference between the presentation of pollen and stigmas in angiosperms II. Herkogamy. *New Zealand Journal of Botany*, **24**: 163-178.

Weiss MR. (2001). Vision and learning in some neglected pollinators: beetles, flies, moths, and butterflies. 171-190.

Wessinger CA. & Rausher MD. (2012). Lessons from flower colour evolution on targets of selection. *Journal of Experimental Botany*, **63**: 5741-5749.

Whitlock MC. (2003). Fixation probability and time in subdivided populations. *Genetics*, **164**: 767-779.

Wilson P, Wolfe AD, Armbruster WS. & Thomson JD. (2007). Constrained lability in floral evolution: counting convergent origins of hummingbird pollination in Penstemon and Keckiella. *New Phytologist*, **176**: 883–890.

Wood TE, Takebayashi N, Barker MS, Mayrose I, Greenspoon PB. & Rieseberg LH. (2009). The frequency of polyploid speciation in vascular plants. *Proceedings of the national Academy of sciences*, **106**: 13875-13879.

10. Conclusiones.

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CONCLUSIONES

1. Los dos linajes de color que aparecen en las poblaciones naturales de *Lysimachia arvensis*, rojo y azul, producen una descendencia homogénea de color salmón cuando se cruzan. Este resultado indica que el carácter "color floral" puede servir como un marcador natural para determinar la tasa de cruce entre linajes en las poblaciones naturales. La escasez de plantas con flores salmón en las poblaciones polimórficas, sugiere algún tipo de aislamiento reproductivo entre los linajes de color.

2. La progenie procedente de autogamia y xenogamia mostró marcadas diferencias en ambos linajes de color. La progenie procedente de autogamia germinó más tarde y necesitó más tiempo para alcanzar la madurez reproductiva que la procedente de xenogamia. Los niveles de depresión por endogamia fueron más altos en el linaje rojo que en el azul en las dos poblaciones estudiadas. A pesar de ello, los niveles de diversidad genética fueron mucho menores en el linaje rojo, lo que sugiere que progenie procedente de autogamia se mantiene en las poblaciones aunque en baja cantidad.

3. Los dos linajes de color mostraron diferencias en su ciclo de vida, germinando antes las semillas del linaje azul, que además mostraron una floración más temprana. También se encontró una fuerte diferenciación genética de los dos morfos, lo que sugiere un bajo flujo génico entre ambos. Las diferencias fenológicas y genéticas sugieren fuertemente que los dos linajes de color están aislados reproductivamente.

4. Las flores de *Lysimachia arvensis* presentan dos tipos secuenciales de hercogamia, una estrategia muy poco frecuente en las plantas con flores. El primer día de apertura, tienen hercogamia lateral con ángulos que oscilan entre 5,6 y 60 grados. El segundo día, el estilo se coloca en posición vertical con respecto a los pétalos y en función de su longitud muestran hercogamia reversa o de aproximación. Aunque ambos tipos de hercogamia fueron eficientes reduciendo la autodeposición del polen, la hercogamia lateral fue mucho más efectiva ya que por encima de 20 grados no se produce autogamia. En la hercogamia vertical, solo la de aproximación impidió la autodeposición del polen. Ambos tipos de hercogamia fueron variables entre plantas y poblaciones, pero mostraron un alto grado de heredabilidad.

5. Los dos linajes de L. arvensis difieren consistentemente en los dos tipos de hercogamia. El linaje rojo mostró un sistema de selfing retrasado, ya que tiene una hercogamia lateral fue fuerte durante el primer día y hercogamia reversa durante el segundo y el tercero. En contraste, el linaje azul fue más variable y mostró poblaciones xenógamas con hercogamia lateral y hercogamia de aproximación, y otras poblaciones autógamas con una hercogamia lateral muy pequeña que permite la deposición automática del polen desde el primer día.

6. Se encontraron diferencias significativas en la expresión de la hercogamia de ambos linajes en función de si se encuentran en poblaciones simpátricas o alopátricas. En poblaciones simpátricas los niveles de hercogamia fueron menores. La hercogamia lateral, la mas eficiente controlando la autodeposición de polen, disminuyó en ambos linajes y la hercogamia de vertical solo lo hizo en el linaje rojo. La variación en el grado de hercogamia fue por tanto asimétrica, siendo más acusada en el linaje rojo. Esta disminución podría estar relacionada con una evolución hacia un sistema reproductivo autógamo que actuaría como mecanismo de refuerzo evitando el cruce entre linajes.

7. El modelo de distribución de nicho mostró un área mayor para el linaje rojo, especialmente en el centro de Europa, y una más pequeña y altamente asociada a la Cuenca Mediterránea para el linaje azul. Los resultados predijeron que en el 62,86% del área natural de la especie solo ocurre uno de los linajes. En las zonas simpátricas los linajes mostraron diferencias fenológicas: el azul floreció antes y durante más tiempo y su flores se mantuvieron abiertas casi dos horas más cada día que el linaje rojo. En esas poblaciones, los polinizadores visitaron preferentemente al linaje azul y realizaron el 75% de las transiciones dentro de cada linaje. Todos estos resultados sugieren fuertemente que los cruzamientos en las poblaciones mixtas son asortativos, con poco flujo polínico entre linajes.

8. Las semillas producidas por hibridación entre linajes tuvieron una tasa de germinación más alta que las procedentes de cruces dentro de los linajes y todas ellas originaron plántulas con una supervivencia similar. Sin embargo, la producción de semillas de estas plantas híbridas fue inferior a la de las plantas no híbridas. Encontramos una gran disminución del fitness de la F2 originada por el cruce entre plantas híbridas, donde el 29% de las plantas fueron completamente estériles y un 20% de las fértiles tuvieron un porcentaje de fructificación muy pequeño.

9. Se ha estimado un alto grado, aunque no completo, de aislamiento reproductivo (RI=0,7855) entre los linajes de *L. arvensis*. Las barreras precigóticas fueron las

principales contribuyentes a dicho aislamiento, especialmente la geográfica, la precedencia polínica y la preferencia de polinizadores. Las barreras postcigóticas fueron mucho menos relevantes, siendo la reducción del éxito reproductivo de la descendencia híbrida de segunda generación la más trascendente. En ese sentido, se observó un incremento en la pérdida de fertilidad y reducción de la producción de semillas, en las sucesivas generaciones de plantas híbridas.

10. Hasta ahora, los estudios filogenéticos que incluían a L. arvensis solo tenían en cuenta a un linaje de color. El estudio filogénetico que hemos llevado a cabo con marcadores cloroplásticos y nucleares indica que los dos linajes de color tanto de *L. arvensis* como de *L. monelli* constituyen actualmente clados diferentes. El linaje azul de *L. arvensis* es hermano de *L. talaverae*, el rojo de *L. arvensis* hermano del linaje rojo de *L. monelli* y el linaje azul de *L. monelli* hermano de *L. foemina*.

11. Nuestros resultados tienen implicaciones taxonómicas que indican que los linajes de color de *L. arvensis* y *L. monelli* deberían ser reconocidos como especies diferentes con identidad morfológica, geográfica y genética. Proponemos para el linaje azul de *L. arvensis* el nombre de *L. loeflingii* J. Jiménez-López, **nom. nov.**, y se mantiene el nombre de *L. arvensis* para el linaje rojo. En el caso de L. monelli, este nombre se matiene para el linaje azul, pero se propone una nueva combinación para el rojo que pasaría a llamarse *L. collina*.

12. El conjunto de resultados obtenidos en esta tesis doctoral indica que el color floral no ha sido el carácter que ha desencadenado el proceso de especiación entre *L. arvensis* y *L. loefflingi*. Sin embargo, dada la disminución tan importante que hemos encontrado en el fitness de la F2 resultante de la hibridación entre estas especies, el color floral podría estar funcionando como mecanismo de refuerzo. El color floral propiciaría el desarrollo de fuertes barreras precigóticas en las poblaciones simpátricas de ambas especies, evitando la aparición de híbridos mal adaptados y manteniendo a *L. arvensis* y a *L. loefflingi* como entidades independientes.

CONCLUSIONS

1. The two colour lineages of *Lysimachia arvensis*, red and blue, originate a homogeneous salmon-colored offspring when they cross. This result indicates that the "floral color" character can be used as a natural marker to determine the crossing rate between lineages in natural populations. The low presence of plants with salmon flowers in polymorphic populations suggests some type of reproductive isolation between color lineages.

2. The progeny from selfing and outcrossing showed marked differences in both lineages of colour. The selfed progeny germinated later and needed more time to reach reproductive maturity than that from outcrossing. The levels of inbreeding depression were higher in the red than in the blue lineage in the two populations studied. Despite this, levels of genetic diversity were much lower in the red lineage, which suggests that progeny from selfing is maintained in the populations although in low quantity.

3. The two color lineages showed phenological differences in their life cycle. Seed germination occurred earlier in the blue lineage and the plants also showed an earlier flowering. A strong genetic differentiation between morphs was found, suggesting a low gene flow between them. The phenological and genetic differences found in this study strongly suggest a reproductive isolation of the two colour lineages.

4. *Lysimachia arvensis* shows both lateral and vertical herkogamy in the same flower, a rare strategy in flowering plants. Lateral herkogamy ranged from 5.6 to 66.5 degrees while vertical herkogamy ranged from reverse to approach herkogamy. Lateral herkogamy was the most important trait limiting self-pollen deposition, such that plants with > 20 degrees of lateral displacement showed almost no self-pollen deposition. Under vertical herkogamy, only approach herkogamy was efficient in reducing self-pollen deposition. Herkogamy traits were constant within plants but variable among plants and populations and showed a high degree of narrow sense heritability.

5. The blue and red lineages of *L. arvensis* differed consistently in both herkogamy traits, a fact that clearly indicates a history of reproductive isolation between lineages. Red flowers showed strong lateral herkogamy, with style-stamen angles generally > 20 degrees. This suggests that red flowers are totally dependent on pollinator activity during the first day of anthesis. However, during the 2nd day of

anthesis, the great majority of red flowers showed no herkogamy, or reverse herkogamy, and there was thus a potential for autonomous delayed self-pollination. In contrast, the blue morph ranged from plants that were mainly outcrossing to plants showing competing selfing, with less marked lateral herkogamy on the first day and approach herkogamy on the second.

6. Values of both herkogamy traits were lower when lineages were found together. The most efficient herkogamy trait controlling selfing, lateral herkogamy, decreased in mixed populations for both lineages and vertical herkogamy only decreased significantly for the red lineage. Thus, the decrease in herkogamy was asymmetric being stronger in the red than in blue morph. The decrease in herkogamy in mixed populations of *L. arvensis* would contribute to reproductive isolation of morphs by increasing selfing as a way to avoid reproductive interference.

7. The niche distribution model showed a larger area for the red lineage, especially in central Europe, and a smaller area for the blue lineage highly associated to the Mediterranean Basin. The results predicted that in 62.86% of the natural area of the species only one of the lineages occurs. In sympatry, both lineages showed phenological differences: the blue flowered before and for a longer time, and its flowers were kept open almost two hours more each day than those of the red lineage. In sympatric populations, pollinators preferentially visited blue flowers and 75% of the transitions occurred within lineages. All these results strongly suggest assortative pollination in sympatry, with reduced pollen flow between lineages.

8. Seeds produced by between-lineage hybridization had a higher germination rate than within-lineages, and all of them originated seedlings with similar survival rate. However, the seed production of hybrid plants was lower than that of the non-hybrid plants. We found a large decrease in the fitness of the F2 coming from the cross between hybrid plants, as 29% of the plants were completely sterile and 20% of the fertile plants had a very low fruiting percentage.

9. A high degree of reproductive isolation has been estimated among L. arvensis lineages (RI = 0.7855). Prezygotic barriers were the main contributors to this isolation, mainly geographic distribution, pollen precedence and pollinator preferences. Postzygotic barriers were much less relevant, being the reduction of the reproductive success of the second-generation hybrid offspring the most relevant. In fact, an increase in the loss of fertility and reduction of seed production was observed in the successive generations of hybrid plants.

10. So far, phylogenetic studies on *Lysimachia arvensis* only included one of the colour morphs in the sampling because so far both morphs have been considered a sole species. The phylogenetic study carried out in this thesis by plastidic and nuclear molecular markers clearly indicates that colour lineages of both *L*. *arvensis* and *L*. *monelli* appear in different clades. The blue lineage of *L. arvensis* is related to *L. talaverae*, the red lineage of *L. arvensis* is related to the red lineage of *L. monelli* and the blue lineage of *L. monelli* is related to *L. foemina*.

11. Our results have important taxonomic implications, as they indicate that color lineages of both L. arvensis and *L*. *monelli* are, in fact, different taxa. We propose the name of *L. loefflingi* J. Jiménez-López, **nom. nov.** for the blue lineage of *L*. *arvensis*, maintaining the name of *L*. *arvesis* for the red lineage. The blue lineage of *L*. *monelli* maintains that name but we propose a new combination for the red lineage that could be named *L*. *collina*.

12. The set of results obtained in this doctoral thesis indicates that the trait "floral colour" has not driven the speciation process between *L*. *arvensis* and *L*. *loefflingi*. However, given the significant decrease in the fitness of the F2 resulting from the hybridization between these species, flower colour could be acting as a "reinforcement mechanism". Reinforcement generates selection favoring the evolution of stronger prezygotic reproductive barriers between emerging species. Flower colour markedly contributes to prezygotic barriers between *L*. *arvensis* and *L*. *loefflingi* in sympatric populations, avoiding the appearance of maladapted hybrids and contributing to the maintenance of these species as independent entities.